

Gene Mutations in Primary Ciliary Dyskinesia Related to Otitis Media

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Abstract Otitis media with effusion (OME) is the most common cause of conductive hearing loss in children and is strongly associated with primary ciliary dyskinesia (PCD). Approximately half of the children with PCD require otolaryngology care, posing a major problem in this population. Early diagnosis of PCD is critical in these patients to minimise the collateral damage related to OME. The current gold standard for PCD diagnosis requires determining ciliary structure defects by transmission electron microscopy (TEM) or clearly documenting ciliary dysfunction via digital high-speed video microscopy (DHSV). Although both techniques are useful for PCD diagnosis, they have limitations and need to be supported by new methodologies, including genetic analysis of genes related to PCD. In this article, we review classical and recently

associated mutations related to ciliary alterations leading to PCD, which can be useful for early diagnosis of the disease and subsequent early management of OME.

Keywords Primary ciliary dyskinesia · Otitis media · Kartagener syndrome · Ciliopathies · Genetics · Cilia · Gene mutations

Introduction

Primary ciliary dyskinesia (PCD, OMIM 242650) is a congenital respiratory disease in which the respiratory cilia are immotile, dysmotile or both [1, 2]. The PCD phenotype is characterised by

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impaired mucociliary clearance, which is believed to be responsible for chronic lung, sinus and middle ear diseases. PCD includes a heterogeneous group of genetic disorders, affecting 1/20,000–60,000 individuals [3]. Approximately 50 % of these patients have situs inversus (SI), a coexistence known as Kartagener syndrome (KS, OMIM 244400) [4]. Most of the males affected by this disease are infertile, as PCD is associated with abnormal sperm flagella motility [5].

Otitis media with effusion (OME) is the most common cause of conductive hearing loss in children, affecting 10–30 % of all children aged 1–3 years [6–8]. When OME is present, it requires otolaryngology care in ~50 % of the children with PCD, posing a major problem in this population [9]. Early treatment of OME in these children is essential to mitigate the effects of hearing loss, speech and language development impairment, recurrent acute otitis media, and chronic otitis media. Although there is controversy regarding the appropriate clinical management of the disease, such as antibiotics and hearing aids versus surgical intervention, its effectiveness increases the earlier treatment begins [6, 9, 10]. While otitis media is influenced by genetics, there has only been one family-based linkage genetic study. This study was performed by Casselbrant et al. in 2009 and included 403 Caucasian families (1,431 genotyped individuals, 377 affected sibling pairs) and 26 African-American families (75 genotyped individuals, 27 affected sibling pairs). They found a significant linkage peak on chromosome 10q22.3 and on 17q12, but the principal limitation was the use of an Affymetrix 10 k SNP array, which contained only 10,000 SNPs across the genome [11]. The heterogeneity of OME and comorbidity with other complex pathologies, including PCD, make it reasonable to isolate OME patients for genetic studies based on the coexistence of other disorders.

The current gold standard for PCD diagnosis requires determining the ciliary structure defects by transmission electron microscopy (TEM) or clearly documenting ciliary dysfunction via digital high-speed video microscopy (DHSV) [12, 13]. Although both techniques are complementary and useful to PCD diagnosis, they have limitations. TEM is limited because 15 % of currently diagnosed PCD patients have no ultrastructural defects [14–17], and many do not easily demonstrate all primary ultrastructural defects, especially those affecting the composition and distribution of the internal dynein arms (IDAs). DHSV is limited because diverse agents, including respiratory pathogens [18, 19] and chemical agents, such as tobacco smoke and pollutants, can alter ciliary beat activity; thus, PCD can only be confirmed after culture of airway ciliated cells [13]. Although there are diagnostic tests, including nasal nitric oxide testing or immunofluorescence of ciliary proteins, new complementary diagnostic tools are needed to accelerate the treatment of PCD patients [20].

Recent studies have identified PCD causing mutations in several genes encoding structural and/or functional proteins in

cilia. These genes include outer dynein arms (ODAs) such as *DNAH5*, *DNAI1*, *DNAI2*, *DNAL1* and *TXNDC3* [21–23, 24•, 25], genes involved in ODA assembly such as *DNAAF1*, *DNAAF2*, *DNAAF3*, *CDC103*, *HATR2* or *LRR6* [26–30], radial spoke genes *RSPH4A* and *RSPH9* [31], nexin-dynein regulatory complex genes *CCDC39* and *CCDC40* [32, 33•], and the central pair apparatus gene *HYDIN* [34••]. Some authors anticipate that genetic testing for PCD will soon become the gold standard diagnostic test in a growing number of cases [20]. However, the complexity of cilia, which involve more than 200 different polypeptides, heterogeneity of mutations and the fact that several mutations result in the same altered phenotype complicate this assumption.

In this review we have focussed on the mutations described for the different proteins involved in PCD, their potential use as complementary diagnostic tools to improve the early detection of PCD, and consequent palliative treatment to minimise otological and respiratory complications.

Normal Cilia Structure

Cilia are strong, conserved complex structures, related to the flagella of sperm and protozoa, that protrude from the apical surface of various eukaryotic cells [35]. They are composed of >200 different polypeptides and can be divided into two types: primary and epithelial cilia [36]. Primary cilia can be categorised as sensory, immotile cilia that act as mechanosensors or nodal motile cilia involved in lateral determination during embryogenesis [37–39]. Epithelial cilia are hair-like appendages present in the respiratory tract, female oviduct, male ductuli efferentes and the ependymal cells of the brain. These cilia are motile and possess an intrinsic ciliary beat pattern [36].

The Basal Body

The basal body is a specialised centriole found at the base of each cilium or flagella that organises ciliogenesis and anchors the cilia in a specific orientation [40]. The syntheses of all the structural and functional elements of cilia occur in the cytoplasm and are transported through the cilia in an anterograde and retrograde manner in a process essential for the construction and maintenance of the cilia. These proteins dock at the basal body while awaiting transport to the cilia via microtubule-associated kinesin and dynein [41].

Microtubules

The axoneme is the central core structure of cilia and exists as α and β tubulin heterodimers arranged in protofilaments to form microtubules. Protofilaments of respiratory cilia are arranged in a classic 9+2 pattern of nine peripheral microtubule doublets

surrounding a pair of central microtubules. Each of the central pair of microtubules is composed of 13 protofilaments containing 23 unique polypeptides, while the outer microtubule doublets consist of the A (13 protofilaments) and B (11 protofilaments) microtubules [36]. By contrast, the protofilaments of nodal cilia are arranged in a 9+0 pattern with nine peripheral microtubule doublets without the central pair [36].

Dynein Arms

Dynein arms are the molecular motors that permit cilia movement. They are composed of dynein chains and are attached to the A microtubule of each of the microtubule pairs by the tubulin. Dynein arms interact with the β tubulin of the adjacent B microtubule in an ATP-sensitive manner such that ATP hydrolysis causes movement of the head of the dynein arm along the B microtubule and the coordinate sliding of the microtubules relative to each other generates the ciliary bend [36, 42]. Dynein arm complexes are composed of heavy, intermediate and light molecular weight dynein chains in addition to at least ten other polypeptides [43]. The ODAs are positioned proximal to the ciliary membrane, while the IDAs are proximal to the central apparatus of the A microtubule and spaced in specific linear repeats of 96 and 24 nm, respectively [36].

Central Pair Apparatus

The central pair apparatus is composed of the two central microtubules with their associated projections, which include the central pair projections, the central pair bridges linking the two tubules, and the central pair caps attached to the distal or plus ends of the microtubules [44]. At least 23 polypeptides, in addition to tubulin, comprise the central apparatus. Ten of them are unique to one central microtubule, while seven are unique to the other microtubule. Therefore, the projections of both microtubules are biochemically and structurally different [45].

The central apparatus plays an important role in the conversion of dynein-induced microtubule sliding into complex ciliary and flagellar waveforms through interactions with the radial spokes [44]. This structure rotates once per beat, and as it rotates, a signal is transduced from the central pair to the dynein arms via the radial spokes, which convert a simple symmetric bend into the asymmetric bends required for forward swimming [46].

Radial Spoke Complexes

The radial spoke is a complex of at least 23 different polypeptides that functions as a mechanochemical transducer between the central pair apparatus and the peripheral dynein arms [40]. It has a T-shaped structure composed of an

elongated stalk that is anchored to the A microtubule of a peripheral doublet and an orthogonal head thought to have transient contacts with the central apparatus [47, 48].

Nexin Links

The nexin link was identified originally in 1963 by Gibbons in cross section and later called the circumferential link. They are elastic elements involved in the conversion of sliding to bending [49]. Their identity had been a mystery for 50 years. However, Heuser et al. demonstrated recently that nexin links are the dynein regulatory complex and that there are connections between them and other axonemal proteins, indicating they are a central hub regulating the cilia and flagella beat [50].

Normal Ciliary Activity

Two distinct types of motility are representative of 9+2 and 9+0 motile cilia. The beat pattern of 9+2 cilia occurs in a waveform, having a forward effective stroke and a return non-effective stroke. The direction of the stroke is controlled by the orientation of the central microtubules. On the other hand, motile nodal 9+0 cilia beat with a vertical motion. This type of motility directs nodal flow leftward across the node, which is necessary for proper left/right asymmetry [39, 51]. Each of the epithelial cells has approximately 200 motile 9+2 cilia that move with both intra- and intercellular synchrony. Ciliary beat frequency ranges from 8–20 Hz under normal situations, but can be accelerated by exposure to irritants such as tobacco smoke [52] through a mechanism involving nitric oxide (NO) [53, 54]. Ciliary beat frequency can also be accelerated by increases in intracellular calcium fluxes [55, 56].

Structural components of cilia are involved in various stages of cilia movement. The central pair of microtubules interacts with the radial spoke to selectively activate subsets of dynein arms. The relative sliding of the microtubule doublets generates the cilia beat with the ODAs controlling the beat frequency and the IDAs controlling the waveform. Nexin links regulate the dynein activity in response to ATP concentration changes and altered nucleotide and salt concentrations [36].

Genetics

Axonemal structure is composed by >200 different polypeptides. In principal, a mutation in any of these proteins can alter ciliary activity and result in a dyskinetic phenotype. Accordingly, PCD is a genetically heterogeneous disorder that poses challenges in terms of defining causative genes. Although conventional family genome-wide linkage studies

have failed to identify PCD-causing genes, other methodologies, including homozygosity mapping or exon analysis using next-generation sequencing, have proved useful for identifying the mutations that cause the PCD phenotype.

Mutations in Genes Encoding Axonemal Dyneins

Several mutations in axonemal encoding genes have been associated with PCD alterations. These include both heavy and intermediate dynein and comprehend complex dynein arm defects with a total or partial lack of ODAs [57, 58].

The dynein axonemal intermediate chain 1 (*DNAI1*) is located in the p21-23 region of chromosome 9 and encodes a 699-amino-acid protein. Mutations in *DNAI1* have been found in 10 % of PCD patients, including those with Kartagener syndrome [23, 59–61]. Mutations in *DNAI1* have been associated with a lack of ODAs and account for ~14 % of those patients. In 2006, Zariwala et al. sequenced the entire coding region of 179 families, whereby 91 % of the affected patients had recurrent otitis media and 9 % of studied families had different mutations in *DNAI1* [61].

The cytogenetic band 5p15.2 of chromosome 5 encodes dynein heavy chain 5 (*DNAH5*). This gene spans 250 kb, comprises 80 exons, and is the human ortholog of the *Chlamydomonas* γ -axonemal heavy chain of the ODAs [62]. Omran et al. analysed a large inbred Arab family with PCD and proposed this as a candidate gene for PCD [63], which has mutations in 49 % of families with documented ODA defects [64]. *DNAH5* mutations have been found only in patients with partial or total loss of ODAs that may or may not be associated with left-right asymmetry anomalies [34•, 64].

Another dynein related to PCD is the dynein axonemal intermediate chain 2 (*DNAI2*). This gene contains 14 exons and is located at 17q25.1. *DNAI2* mutations have been found in 2 % of PCD families and in the 4 % of PCD families with documented ODA defects [64].

Dynein axonemal light chain 1 (*DNAL1*) is another protein associated with the development of DCP with absence of ODAs and complete SI, early neonatal respiratory distress and chronic sino-pulmonary infection leading to severe morbidity [24•].

Not all dynein gene mutations lead to ultrastructure alterations involving ODAs or IDAs. This is the case for dynein axonemal heavy chain 11 (*DNAH11*), which is located at 7p21 and comprises 82 exons. Mutation in this gene is frequent in PCD patients without ciliary ultrastructural defects [65•, 66].

Mutations in Components of the ODA Docking Complex

Recently, Knowles et al. sequenced the complete exoma of six unrelated individuals with isolated ODA defects, the most common defect in PCD patients [67•]. They analysed a mutation in one of the patients in *CCDC114*, which is one of

the components of the ODA docking complex. They also found mutations in 4 of 67 patients showing ODA alterations, while no mutations were found in 33 PCD-diagnosed patients without ODA defects. Other authors have found mutations in *CCDC114* associated with PCD [68•]. *CCDC114* is located on chromosome 19q13.3 and the human gene is an ortholog of *Chlamydomonas* *DCC2*, which encodes an axonemal outer dynein arm-microtubule-docking complex subunit [69]. This gene is expressed in motile ciliated tissues, including in the nasal cavity epithelium and brain ventricles [57]. Moreover, it has the same expression pattern as *DNAI1* and is expressed in equimolar amounts [70, 71]. Interestingly, although *CCDC114* localises along the entire length of human cilia and its deficiency causes the complete absence of ciliary ODAs, fertility is not affected in patients carrying mutations in this gene [68•]. There may be some functional redundancy of *CCDC114* in sperm with other genes, such as *CCDC63*, but this question remains under investigation.

Mutations in Genes Involved in the Cytoplasmic Preassembly of Dynein-arm Components

Abnormal cytoplasmic preassembly of dynein-arm components can dramatically affect the structure and function of cilia. Genetic mutation of different genes can explain this disturbance, as has been reported for the leucine-rich repeat-containing protein 50 (*LRRC50*), which is the human ortholog of the *Chlamydomonas* *ODA7* gene. *ODA7* is necessary for dynein preassembly and may participate in a structural link between inner- and outer-row dyneins [72]. Interestingly, two different papers described an alteration in ODA and IDA defects in individuals carrying mutations in this gene. In one of them, the authors identified mutations in *LRRC50* using high-throughput technologies, such as Affymetrix SNPs arrays, while the other report focussed specifically on this gene because of previous results implicating it in PCD [28, 73]. The authors also analysed the expression pattern of *LRRC50*, finding a typical expression pattern of PCD-related genes in the testis and trachea [28]. Patients carrying mutations in *LRRC50* also have either totalis or solitus SI.

Another dynein-interacting protein related to PCD is the dynein axonemal assembly factor 2 (*DAAF2*), also known as KTU. The protein encoded by this gene specifically interacts with Hsp70 and *DNAI2*, and the mutation causes a PCD phenotype in *Chlamydomonas*, mice and humans [27].

Recently, Mitchison et al. associated the previously uncharacterised protein *DNAAF3* with the preassembly of dyneins into complexes prior to their transport into cilia. They found that mutation of *DNAAF3* in zebrafish disrupted dynein arm assembly, causing PCD phenotypes including hydrocephalus and laterality malformations. Moreover, they identified loss of function mutations in patients from families with SI and defects in the assembly of IDAs and ODAs [26].

Dyslexia susceptibility 1 candidate (*DYX1C1*) was initially identified as a candidate for dyslexia [74]. Tarkar et al. found that mutations in this gene cause laterality and ciliary motility defects in zebrafish and mice. They analysed DNA from 1 highly inbred Irish family, 5 US families and Australian PCD families, and 105 individuals with PCD combined with ODA and IDA defects. They found nine different mutations, seven of which were predicted to cause premature protein termination in 15 of the families studied. They reported that *DYX1C1* was localised in the cytoplasm of respiratory epithelial cells and interacts with the cytoplasmic ODA and IDA assembly factor *DNAAF2* [75•].

More candidate PCD genes involved in the cytoplasmic preassembly of dynein-arm components are being described. One example is *ZMYND10*. Moore et al. used whole-exome and candidate-gene Sanger re-sequencing to describe mutations in 6 of 38 PCD patients, demonstrating that mutation of this gene causes dynein-arm loss and complete cilia immobility [76•].

Mutations in Components of the Central Apparatus

Another example of PCD without structural axonemal alteration is that caused by mutations in the axonemal central pair protein *HYDIN*. Recently Olbrich et al., using Affymetrix 10 k SNPs arrays, found a 21.8-Mb region of homozygosity shared in chromosome 16q in a German family. This region included 250 genes, 2 of which were of interest for PCD: *DYNLRB2*, which was discarded because the mutant resulted in normal ultrastructure, and *HYDIN*, which was related to a hydrocephalus model. Exon sequencing of *HYDIN* identified the mutation c.3985G>T in three affected siblings, which produced aberrant alternative splicing, as well as the nonsense mutation c.922A>T in three individuals from three Faroe Island PCD families. Individuals carrying this alteration had normal axonemal composition as well as normal situs composition. None of them exhibited congenital heart disease or hydrocephalus. DHSV studies demonstrated that *HYDIN* mutant respiratory cilia and sperm flagella exhibited abnormal axonemal beating and the authors' concluded that this was due to abnormal central apparatus function [34••].

Mutations in Genes Affecting IDA and Axonemal Organisation

In 2011, Merveille et al. examined five Old English Sheepdogs with chronic airway inflammation and SI. Electron microscopy revealed the absence of eccentric central pairs, abnormal radial spokes and nexin links. Using the Affymetrix canine array they found a 15-Mb segment of autozygosity containing *CCDC39*, which is involved in ciliary defects found in *Chlamydomonas*, mice and zebrafish studies [32, 77]. They sequenced *CCDC39* exons in 53 cases of PCD

patients with the same structural alteration observed in Bobtails and found alterations in 38 % of families analysed. Functional studies indicated that this gene is located to ciliary axonemes and is essential for the assembly of IDAs and the dynein regulatory complex [32].

Another gene related to defects in IDAs and central pair positioning is the coiled-coil domain containing 40 gene *CCD40*. Functional studies performed in mouse and zebrafish embryos demonstrated the implication of this gene in the control of laterality. Mutations in *CCDC40* resulted in SI and heterotaxia. Patients carrying mutations in *CCDC40* have *situs solitus* (32 %), *situs inversus* (68 %), misplaced and/or duplicated central tubules, misplaced peripheral doublets and abnormal motility [33•].

Mutations in *CCDC39* and *CCDC40* have been found in 2 of 43 patients with congenital heart disease and heterotaxia [78]. Genetic studies performed in patients with IDA and axonemal disorganisation revealed that *CCDC39* and *CCDC40* are the major cause of PCD with these alterations [79••, 80].

Mutation in Genes Involved in Sensory Cilia

Although PCD is commonly inherited as an autosomal recessive trait, rare cases of pedigrees showing autosomal dominant or X-linked modes of inheritance have been reported [81–86]. Patients with this type of inheritance display syndrome phenotypes. One example was found in a family with PCD and X-linked recessive mental retardation and macrocephaly, caused by mutation in the oral facial digital type 1 syndrome gene (*OFD1*) [85]. Another example was found in the overlapping of PCD and the X-linked form of the retinitis pigmentosa (XLRP) [87]. Although the 70–80 % of the familial and the 10–20 % of sporadic cases of XLRP are due to mutations in the retinitis pigmentosa GTPase regulator (*RPGR*), all the genetically diagnosed cases of PCD overlapping with this disease have been related to mutations in *RPGR*. *RPGR* is expressed in the connecting cilium between the inner and outer segment of photoreceptors and in the transition zone of respiratory cilia [88, 89]. Recently, Bukowy-Bieryłło et al. found that mutation in *RPGR* in a Polish patient with syndromic PCD did not induce a lack of ultrastructural defects, but did result in a severe disturbance of cilia orientation with subsequent reduction in cilia motility [90••].

Conclusions

OME is the most common cause of conductive hearing loss in children, affecting 10–30 % of all children aging 1–3 years. Approximately half of the children with PCD required otolaryngology care for management of middle ear disease. In PCD, OME is a consequence of chronic alteration of cilia activity, which generates an exudate in the middle ear and is common

to all patients, including children. In most cases when OME is diagnosed, surgical treatment is necessary. For these patients an early diagnostic of PCD is essential prior to treatment, preferably as soon as the first manifestation of symptoms related to OME appear, to improve hearing and mitigate the effects of hearing loss on speech and language development. We have provided a review of the principal related PCD genes and sequence analysis of these genes can provide an early diagnostic indicator for PCD, supporting TEM and functional DHSV, especially in those cases free from ultrastructural defects.

Compliance with Ethics Guidelines

Conflict of Interest Manuel Mata, Lara Milian, Miguel Armengot and Carmen Carda declare that they have no conflict of interest.

Human and Animal Rights and Informed Consent This article does not contain any studies with human or animal subjects performed by any of the authors.

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- Of major importance

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