

The Emerging Genetics of Primary Ciliary Dyskinesia

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Primary ciliary dyskinesia (PCD) is an autosomal recessive, rare, genetically heterogeneous condition characterized by oto-sino-pulmonary disease together with situs abnormalities (Kartagener syndrome) owing to abnormal ciliary structure and function. Most patients are currently diagnosed with PCD based on the presence of defective ciliary ultrastructure. However, diagnosis often remains challenging due to variability in the clinical phenotype and ciliary ultrastructural changes. Some patients with PCD have normal ciliary ultrastructure, which further confounds the diagnosis. A genetic test for PCD exists but is of limited value because it investigates only a limited number of mutations in only two genes. The genetics of PCD is complicated owing to the complexity of axonemal structure that is highly conserved through evolution, which is comprised of multiple proteins. Identifying a PCD-causing gene is challenging due to locus and allelic heterogeneity. Despite genetic heterogeneity, multiple tools have been used, and there are 11 known PCD-causing genes. All of these genes combined explain approximately 50% of PCD cases; hence, more genes need to be identified. This review briefly describes the current knowledge regarding the genetics of PCD and focuses on the methodologies used to identify novel PCD-causing genes, including a candidate gene approach using model organisms, next-generation massively parallel sequencing techniques, and the use of genetically isolated populations. In conclusion, we demonstrate the multipronged approach that is necessary to circumvent challenges due to genetic heterogeneity to uncover genetic causes of PCD.

Keywords: cilia; dynein; Kartagener syndrome; dextrocardia; heterotaxy

Defective ciliary or flagellar structure and function leads to primary ciliary dyskinesia (PCD) (MIM 242650), which has several clinical manifestations, including chronic rhinosinusitis, otitis media, bronchitis, pneumonia, bronchiectasis, male infertility, and reduced fertility in female patients (1–3). Approximately 50% of patients with PCD have situs inversus totalis (termed Kartagener syndrome, MIM244400), and situs ambiguus occurs in at least 6% of patients with PCD (4). PCD is a rare, genetically heterogeneous, autosomal recessive disorder with an estimated incidence of approximately 1 in 16,000 to 20,000. There

is no gender, racial, or geographical bias; however, certain geographically isolated communities or ethnic groups may have a higher prevalence of PCD due to inbreeding (5, 6). Further details concerning the clinical manifestations and diagnostic testing for PCD are discussed in accompanying articles in this issue of the *Journal*.

In this session, “The Emerging Genetics of Primary Ciliary Dyskinesia,” Dr. Maimoona Zariwala from the University of North Carolina at Chapel Hill began by summarizing the current knowledge about the genetics of PCD and emphasizing that the latest exome-capture and massively parallel sequencing technologies will assist in unraveling the genetic basis of PCD. Dr. Heymut Omran from the Universitätsklinikum Münster followed with a presentation describing two novel PCD-causing genes that were identified using a candidate gene approach. Dr. Thomas Ferkol from Washington University in St. Louis concluded the session by discussing the role of genetically isolated populations, such as the Amish, in identifying novel PCD genes and possible studies of genotype–phenotype correlations in this cohort. We summarize all three presentations in this article and discuss areas that require further investigation. This article begins with the presentation by Dr. Omran, followed by the presentations by Dr. Ferkol and Dr. Zariwala. Furthermore, some of the data presented by Dr. Omran (7, 8) and Dr. Ferkol have been previously published (5).

OVERVIEW OF PCD GENETICS

Due to genetic, phenotypic, and ciliary ultrastructural heterogeneity, the diagnosis of PCD remains challenging. Although certain diagnostic techniques are available, they are time- and labor intensive and require special training or reagents and hence are performed only at a few specialized centers. Ciliary ultrastructural analysis is useful if axonemal defects are present, but 28% of patients with PCD have normal axonemal ultrastructure (9), which confounds the diagnosis. In fact, mutations in *DNAH11*, a gene that encodes dynein axonemal heavy chain 11, occur in a subset of patients with PCD who present with normal ciliary ultrastructure; therefore, genetic testing is critical for the diagnosis in such cases. Although a PCD clinical genetic test exists in the United States, it only interrogates a few mutations in *DNAI1* and *DNAH5*, providing limited value (1–3). More comprehensive testing is offered internationally.

The genetics of PCD is complicated, which reflects the evolutionarily conserved and complex axonemal structure, which is comprised of multiple proteins. Ciliary structure is reviewed by Ostrowski and colleagues in this issue of the *Journal* (pp. 423). PCD-causing mutations have been identified in 11 genes, encoding a variety of axonemal components, including the two latest genes described in this session (Table 1) (1–3, 7, 8). Mutations in two genes (*DNAI1* and *DNAH5*) that code for ciliary outer dynein arm proteins are the most common genetic causes of PCD (18–30% of PCD), but mutations in the remaining genes are relatively uncommon. Together, mutations in all 11 genes explain approximately 50% of all cases of PCD (reviewed in References 3, 10, and 11).

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TABLE 1. GENES THAT CAUSE PRIMARY CILIARY DYSKINESIA

| Human Gene | Axonemal Component | Ciliary Ultrastructure of Patients with PCD with Biallelic Mutations | OMIM* |
|---------------|--------------------------|--|----------------------|
| <i>DNAH5</i> | ODA-HC | ODA defects | 603335 |
| <i>DNAH11</i> | ODA-HC | Normal | 603339 |
| <i>DNAI1</i> | ODA IC | ODA defects | 604366 |
| <i>DNAI2</i> | ODA IC | ODA defects | 605483 |
| <i>TXNDC3</i> | ODA LC/IC | Partial ODA defects [†] | 607421 |
| <i>KTU</i> | Cytoplasmic [‡] | ODA+IDA defects | 612517 |
| <i>LRRC50</i> | Cytoplasmic [‡] | ODA+IDA defects | 613190 |
| <i>RSPH4A</i> | RSH | Tx and CP defects | 612647 |
| <i>RSPH9</i> | RSH | CP defects or normal | 612648 |
| <i>CCDC39</i> | DRC | Microtubule disorganization [§] | Current [¶] |
| <i>CCDC40</i> | DRC | Microtubule disorganization [§] | Current [¶] |

Definition of abbreviations: CP = central pair; HC = heavy chain; IC = intermediate chain; IDA = inner dynein arm; LC = light chain; ODA = outer dynein arm; PCD = primary ciliary dyskinesia; RS = radial spoke head; Tx = transposition.

* OMIM web resource: <http://www.ncbi.nlm.nih.gov/Omim/>

[†] Partial defect with approximately two thirds of cilia with shortened or absent and one third with normal ODA.

[‡] Cytoplasmic proteins required for the dynein arms assembly.

[§] Microtubule disorganization characterized by reduced number of IDA, eccentric CP, abnormal alignment of outer doublets, and occasional displacement of outer doublet.

[¶] Recent studies presented in this conference and no OMIM numbers assigned yet.

Identifying PCD-causing genes is challenging due to locus heterogeneity; hence, conventional family-based, genome-wide linkage studies have not been successful (12). Candidate gene testing and homozygosity mapping alone or in combination have resulted in the identification of multiple PCD-causing genes, including the two novel genes reported in this session. However, these methods require a large inbred kindred and the ability to select pertinent candidate genes. Recent advances in next-generation, high-throughput (massively parallel) sequencing technology provides opportunities to screen multiple loci simultaneously to define the etiology of a genetic disorders (13). This technology is promising for genetically heterogeneous disorders such as PCD. Defining additional PCD-causing genes will be helpful for genetic diagnosis and for the development of a genetic screening test using massively parallel sequencing technology.

NOVEL PCD VARIANTS CHARACTERIZED BY ABNORMAL DYNEIN REGULATORY COMPLEX FUNCTION

PCD-causing genetic defects have been mainly characterized as defects in outer dynein arms (ODA) or the ODA+Inner dynein arms (IDA) and the central pair apparatus. Recently, a novel group of PCD variants was identified in patients with characteristic changes in ciliary ultrastructure, including a reduced number of inner dynein arms, eccentric central pairs, abnormal alignment of outer doublets, and occasional displacement of outer doublets. These mutant respiratory cilia have a beating pattern with severely reduced amplitude and very rigid axonemes that appear to be very stiff and straight. The clinical phenotype of this novel variant PCD is compatible with classic PCD phenotype, including chronic airways disease with bronchiectasis, randomization of left-right asymmetry, and immotile sperm tails.

Based on candidate approaches in dog, fish, and mouse models of variant PCD, the genes *CCDC39* and *CCDC40* were identified as candidate genes for PCD (7, 8). Mutational analyses of patients with PCD identified recessive loss-of-function mutations in *CCDC39* and *CCDC40* in 13 and 60% of affected patients, respectively (7, 8). High-resolution immunofluorescence analyses of mutant respiratory cilia from patients with *CCDC39* and *CCDC40* found an absence of the dynein regulatory complex (DRC) component, GAS11, and the inner dynein arm light chain, DNALI1, from the ciliary axonemes. Both genes encode for

uncharacterized, coiled-coil, domain-containing proteins. FAP59, the orthologue of *CCDC39* in *Chlamydomonas*, was predicted to be essential for ciliary motility because orthologues do not exist in nonciliated organisms (14). *CCDC39* is normally localized along the whole respiratory ciliary axoneme and is absent from cilia in patients with *CCDC39* and *CCDC40* mutations. Based on these findings, it is very likely that these two proteins interact with each other. It is possible that both *CCDC* proteins physically interact with other axonemal components and serve as a structural part of the axoneme, possibly as a new DRC component. This is consistent with findings that mutations in genes encoding DRC components in *Chlamydomonas*, such as *PF2*, cause a similar ultrastructural phenotype in flagella, including the failure to assemble the DRC and the DNALI1-containing inner dynein arms (15–17). As proposed for *PF2*, *CCDC39* and *CCDC40* could therefore encode for proteins involved in the stability of the DRC complex by interacting with one or several DRC subunits. Alternatively, it is possible that *CCDC39* and *CCDC40* are important for the cytoplasmic pre-assembly of axonemal proteins or axonemal targeting and transport of the axonemal components GAS11 and DNALI1 because they were also found in the apical cytoplasm. However, nothing is known about the process of cytoplasmic preassembly and axonemal targeting and delivery of DRC complexes.

PCD GENES: LESSONS FROM THE AMISH

The Old-Order Amish Mennonite Church communities present unique opportunities for genetic research. In the seventeenth century, the Amish and Mennonite churches were established in Switzerland during the Reformation, and many members emigrated to the United States in the eighteenth and nineteenth centuries to flee religious persecution. First settling in rural Pennsylvania, close-knit agrarian Amish communities have spread across North America. Amish settlements in the Midwest have greatly increased in size and number during the past century. These communities maintain strict adherence to religious customs. Because members rarely marry outside the church, consanguinity is common, resulting in closed populations with a high frequency of genetic disorders. Intermarriage, relative isolation, and detailed record-keeping make Amish communities ideal for genetic studies. We found several children and adults in geographically dispersed Amish communities with PCD (5) and hypothesized that addi-

tional evaluation would confirm a high frequency of PCD in these communities and identify a founder mutation.

Our studies identified nine Amish subjects (within seven subfamilies) who had an unequivocal diagnosis of PCD based on clinical history (*situs inversus totalis*, neonatal respiratory distress, recurrent pneumonia, chronic sinusitis, or male infertility), reduced nasal nitric oxide values, and ultrastructural defects of the ciliary axoneme consistent with the diagnosis (ODA defect) (3, 5, 18). Initially, analyses for known mutations in *DNAI1* and *DNAH5* were performed on affected individuals and family members. Three affected patients from three subfamilies were heterozygous for the *DNAI1* (IVS1+2_3insT) mutation (5, 19), but full coding gene sequencing did not reveal the second mutant allele. Haplotype analysis of *DNAI1* failed to reveal evidence of concordance for the second allele in any of these patients, indicating another gene as causative. A single subfamily harbored a mutation on exon 63 (10815dT) of *DNAH5*, a founder mutation associated with PCD (20). Thus, we found mutations in two PCD-causing genes in an inbred Amish-Mennonite cohort, but most subjects have an unidentified novel genetic defect.

Intragenic single nucleotide polymorphism (SNP) and micro-satellite genotyping confirmed that *DNAI1*, *DNAH5*, and seven other candidate genes (*DNAH11*, *DNAH7*, *DNAH9*, *DNAI2*, *DNAL4*, *DNAH3*, and *TCTEL1*) were not linked to PCD in the remaining family members. GeneChip Genome-Wide Human SNP Array 6.0 identified a region of interest on chromosomes 12 (88,373,816–88,974,238) and 13 (48,516,791–49,056,026), which included candidate genes associated with ciliary or flagellar defects in patients, or *Chlamydomonas*. Exome sequencing using next-generation technology is underway.

In summary, we discovered that a high prevalence of PCD exists in Amish settlements, likely related to consanguinity inherent in this population. Although a new *DNAH5* mutation was identified in several members of geographically dispersed Amish communities, it did not cause PCD in the majority of subjects studied, which implies that a different disease-causing gene mutation is present in these families. Indeed, this large Amish family had surprising genetic heterogeneity, which could allow us to pursue genotype–phenotype relationships in the community.

EXOME-CAPTURE AND MASSIVELY PARALLEL SEQUENCING FOR PCD

Massively parallel sequencing technology, otherwise known as next-generation sequencing, can be used to capture regions of interest, including known and potential candidate genes for PCD, the ciliome (> 1,000 axonemal genes obtained by proteomic analysis), the exome (all coding exons and splice junctions for all the known genes in the genome, which is approximately 1% of the genome), or the entire genome. This technology is useful in genetically heterogeneous disorders because no prior knowledge of candidate genes or pathways is necessary. Multiple platforms for the capture and sequencing technologies are available (21). This technology produces a massive dataset; therefore, data analysis requires sophisticated bioinformatics tools and availability of the DNA from the family members to validate variants of interest.

Due to the extensive locus heterogeneity of PCD, we used whole-exome capture using Nimblegen (CCDS2008 gene set; Roche NimbleGen Inc., Madison, WI) coupled with paired-end (massively parallel) sequencing and an Illumina GA2X analyzer (Illumina Inc., San Diego, CA) to test for novel genes and mutations in well phenotyped patients with PCD. These patients were subgrouped by ultrastructural defects and included

defective ODA, IDA alone or combined, defective radial spokes and central apparatus, acilia/oligo cilia, and normal ciliary ultrastructure with the compatible clinical phenotype and low levels of nasal nitric oxide. For each patient, we obtained an average of approximately 17,000 base substitutions and approximately 500 insertions or deletions. Preliminary data analysis has revealed biallelic mutations in six unrelated patients in *RSPH9*, *DNAI2*, and *DNAH5* genes, previously known to be causing PCD (20, 22–24). A homozygous loss-of-function mutation was discovered in *RSPH9* in a patient with PCD and normal ciliary ultrastructure. Three unrelated patients with PCD and ODA defects harbored a novel homozygous stop mutation in *DNAI2*. All three patients had Jewish ethnicity, and haplotype analysis suggested a founder effect. Identification of this novel founder mutation suggests that mutations in *DNAI2* may be more prevalent in subjects of Jewish ethnicity. Two unrelated patients with PCD and ODA defects had compound heterozygous mutations in *DNAH5*. One of the *DNAH5* mutations was previously missed by Sanger sequencing. Further analysis of that sample revealed the presence of SNP in one of the PCR primers causing no amplification of the allele carrying SNP (allele dropout). These observations show that mutations can be identified using exome capture and massively parallel sequencing technology. Additionally, mutations may be discovered in a gene that has previously been tested using standard Sanger sequencing and deemed negative. In summary, exome sequencing can define novel disease-causing mutations for genetically heterogeneous disorders such as PCD.

FUTURE DIRECTIONS

In a breakout session entitled “Defining PCD Genes and Gene Mutations through Global Networking,” a working group discussed the following topics: (1) data analysis strategies for next-generation sequencing technologies, (2) using next-generation sequencing in patients born to consanguineous union or families with multiple affected siblings, (3) prioritization of variants identified in next-generation sequencing technologies, and (4) the global effort needed to tackle genetic heterogeneity and reduce duplication of efforts.

Next-generation sequencing technologies interrogate millions of bases in parallel but produce a large number of variants, which need to be validated before it is deemed useful. For topic 1, we did not come up with a final proposal but agreed that massive data analysis has limitations because each of the current methods has caveats with respect to the sequence coverage and data filters. We agreed that stringent filters are useful initially to reduce the number of variants that require validation, but, in many cases, nonstringent filters may be needed to ascertain that the gene of interest is not lost due to high stringency criteria. For topic 2, we recognized that using one affected individual from a family poses challenges with data analysis; hence, multiple affected sibs or a patient with known parental consanguinity may be more useful. It was suggested that using a SNP chip before exome capture might be helpful to identify regions of homozygosity (useful for inbred pedigrees) and regions of concordant genotypes (in an affected sib-pair), which could be used to direct final next-generation sequence data analysis (25). For topic 3, we agreed that multiple variants from exome capture, as well as missense variants and variants of uncertain significance, can be compared with the known genes in the same pathways from the model organisms that can aid with the validation process. For topic 4, we agreed that global efforts are needed to circumvent the problem of genetic heterogeneity. Moreover, it is efficient to test each gene in a large number of patients by collaborating to combine affected

individuals from multiple groups. This approach will help decipher the mutation frequency data from larger cohort, instead of multiple groups working on the same gene with a smaller pool of patients. We also agreed that it would be important to identify funding sources to assist the global effort to treat this rare disorder.

CONCLUSIONS

We have demonstrated that multiple strategies can be used to identify genetic causes of PCD, which is largely a genetically heterogeneous disorder. Candidate gene analysis continues to prove useful in identifying novel genes for PCD as mutations in *CCDC39* and *CCDC40* caused disease due to abnormal DRC function. Furthermore, genetically isolated populations, such as Amish-Mennonites, provide unique opportunities in understanding the genetic basis of complex diseases such as PCD. In addition, the recent advances in next-generation sequencing technologies are very promising and have demonstrated novel genetic mutations in patients with PCD. This technology has the potential to develop a clinical molecular genetic test for PCD by using targeted capture of all known PCD-causing genes and interrogating them simultaneously in large numbers of patients.

WEB RESOURCES

<http://www.ncbi.nlm.nih.gov/Omim/>
<http://rarediseasesnetwork.epi.usf.edu/gdmcc/index.htm>

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