The Role of Molecular Genetic Analysis in the Diagnosis of Primary Ciliary Dyskinesia

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Abstract

Rationale: Primary ciliary dyskinesia (PCD) is an autosomal recessive genetic disorder of motile cilia. The diagnosis of PCD has previously relied on ciliary analysis with transmission electron microscopy or video microscopy. However, patients with PCD may have normal ultrastructural appearance, and ciliary analysis has limited accessibility. Alternatively, PCD can be diagnosed by demonstrating biallelic mutations in known PCD genes. Genetic testing is emerging as a diagnostic tool to complement ciliary analysis where interpretation and access may delay diagnosis.

Objectives: To determine the diagnostic yield of genetic testing of patients with a confirmed or suspected diagnosis of PCD in a multiethnic urban center.

Methods: Twenty-eight individuals with confirmed PCD on transmission electron microscopy of ciliary ultrastructure and 24 individuals with a probable diagnosis of PCD based on a classical PCD phenotype and low nasal nitric oxide had molecular analysis of 12 genes associated with PCD.

Results: Of 49 subjects who underwent ciliary biopsy, 28 (57%) were diagnosed with PCD through an ultrastructural defect. Of the 52 individuals who underwent molecular genetic analysis, 22 (42%) individuals had two mutations in known PCD genes. Twenty-four previously unreported mutations in known PCD genes were observed. Combining both diagnostic modalities of biopsy and molecular genetics, the diagnostic yield increased to 69% compared with 57% based on biopsy alone.

Conclusions: The diagnosis of PCD is challenging and has traditionally relied on ciliary biopsy, which is unreliable as the sole criterion for a definitive diagnosis. Molecular genetic analysis can be used as a complementary test to increase the diagnostic yield.

Keywords: primary ciliary dyskinesia; genetic analysis; diagnostic testing

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Primary ciliary dyskinesia (PCD) is a genetically heterogeneous autosomal recessive disease of motile cilia with an incidence of approximately 1/16,000 live births. Clinical manifestations include chronic bronchitis, neonatal respiratory distress, bronchiectasis, sinusitis, otitis media, situs inversus, and male infertility (1). The differential diagnosis of PCD includes respiratory disease, such as cystic fibrosis, asthma, and interstitial lung disease, and other system diseases, such as gastroesophageal reflux, immunodeficiency, and allergic rhinitis, often delaying the diagnosis, especially in the absence of situs inversus (2).

The diagnosis of PCD combines clinical characteristics, diagnostic investigations, and pathology. However, due to its clinical variability and limitations of current testing modalities, the diagnosis of PCD is often uncertain (3). The gold standard for PCD diagnosis traditionally has been transmission electron microscopy (TEM) of ciliary biopsy showing a ciliary ultrastructural defect (1). Analysis of ciliary beat pattern using video microscopy has also been shown to be helpful in the diagnosis of PCD (1, 2). However, ciliary biopsy poses the problems of being uncomfortable, being available only in a very limited number of tertiary care hospitals, and having a significant false-negative and false-positive rate (4).

A number of research tests, including nasal nitric oxide and radioactive mucociliary clearance studies, help to phenotype patients who have nondiagnostic ciliary pathology (1, 2). However, these specialized diagnostic tests are not offered in most institutions and are not reliable in preschool children, limiting their usefulness for early diagnosis. A major limitation for the understanding of PCD is the lack of a definitive diagnosis in many individuals with a PCD phenotype. With the ongoing discovery of genes associated with PCD, molecular genetic analysis is emerging as an accessible and standardized diagnostic test where more than 50% of PCD patients possess two disease-causing mutations *in trans* (1). Furthermore, genetic testing allows for carrier testing, prenatal testing, and genetic counseling. However, the role and cost-effectiveness of genetic testing in the diagnosis of PCD has yet to be fully evaluated (3).

Motile cilia are composed of nine peripheral microtubule doublets surrounding a central microtubular pair ("9+2" arrangement) along with microtubular-associated proteins, such as inner dynein arms (IDA), outer dynein arms (ODA), radial spokes, and nexin links (1). There are currently 24 genes identified in nonsyndromic PCD, and each is associated with a specific ciliary ultrastructural defect. ODA defects are observed in the majority of patients with PCD (5), and mutations in seven genes (*DNAH5*, *DNAI1*, *DNAI2*, *DNAL1*, *TXNDC3*, *CCDC114*, and *ARMC4*) have been identified in such patients (6–13). Mutations in *LRRC50/DNAAF1*, *KTU/DNAAF2*, *DNAAF3*, *CCDC103*, *HEATR2*, *LRRC6*, *ZMYND10*, and *DYX1C1* cause both ODA and IDA defects (14–22). Central microtubular pair (CP) abnormalities are seen with mutations in *RSPH4A*, *RSPH9*, and *RSPH1* (23, 24). Radial spoke defects associated with *CCDC39* and *CCDC40* have been further characterized to involve IDA and nexin-links (nexin regulatory complexes) (25–27). However, normal cilia or very subtle ciliary ultrastructural abnormalities are observed in patients with mutations in four genes associated with PCD: *DNAH11*, *HYDIN*, *CCDC 164*, and *CCDC65* (28–31). Furthermore, not all ciliary specimens are adequate for TEM, making the diagnosis of PCD solely on the basis of TEM difficult (4, 5).

At the start of our study, 12 genes were known to be involved in PCD and were being offered for clinical molecular genetic analysis. Due to the challenges in the diagnosis of PCD, we undertook a study to compare the diagnostic yield of molecular genetic analysis with that of other diagnostic tools, including ciliary biopsy.

Methods

Patients

Inclusion criteria for this study were patients who have either (*1*) definite PCD based on clinical phenotype plus a recognized ciliary ultrastructural defect, or (*2*) probable PCD based on the clinical phenotype and a low nasal nitric oxide (<100 nl/min) in patients 5 years and older who were able to perform this test reliably using the standard technique of exhalation against a resister (32). The empirical cut point of 100 nl/min was based on the largest previously published case series of patients with PCD and published normative values (33, 34). For patients who were given a clinical diagnosis of PCD with an inconclusive biopsy under the age of 5 years, only those who were able to reliably perform nasal nitric oxide testing at a later age and met our inclusion criteria were included in our study sample to avoid diagnostic misclassification that may result from indiscriminate nasal nitric oxide levels in children younger than age 5 years (35). The patients were evaluated at the Hospital for Sick Children (n = 42) and St Michael's Hospital in Toronto (n = 10). A retrospective chart review was conducted, and collected data elements included personal and family medical history, diagnostic investigations, nasal nitric oxide, the results of the TEM analysis of the biopsies of cilia, and clinical genetic testing.

Mutation Profiling and Analysis

All patients enrolled in this study had clinical genetic testing conducted on 12 genes, mutations that have been implicated in PCD. DNA was extracted from peripheral blood and analyzed at Prevention Genetics, Wisconsin in a Clinical Laboratory Improvement Amendments–approved laboratory. Bidirectional Sanger Sequencing was conducted interrogating all coding exons of each gene, plus 50 base pairs of flanking noncoding DNA on either side of each exon. Genes were tested in a sequential fashion until one mutation was identified. If a second pathogenic mutation was not identified, the next gene in the panel was sequenced. For individuals with no mutation identified, all 12 genes were tested. Genes were analyzed in the following order, *DNAH5* (RefSeq: NM_001369.2), *DNAI1* (RefSeq: NM_012144.2), *CCDC39* (RefSeq: NM_181426.1), *CCDC40* (RefSeq: NM_017950.2), *DNAI2* (RefSeq: NM_023036.4), *DNAH11* (RefSeq: NM_003777.3), *RSPH9* (RefSeq: NM_152732.4), *RSPH4A* (RefSeq: NM_001010892.2), *KTU/DNAAF2* (RefSeq: NM_018139.2), *LRRC50/DNAAF1* (RefSeq: NM_178452.4), *TXNDC3* (RefSeq: NM_016616.4), and *DNAL1* (RefSeq: NM_031427.3). Prioritization was based on previous reports of mutation frequency for each gene (1). For example, *DNAH5* is reported to be the most frequently mutated gene (15–21%), followed by *DNAI1* (2–9%), *CCDC39* (2–10%), and *CCDC40* (1–8%) (1). *DNAI2* is reportedly mutated in approximately 2 to 4% of patients with PCD, and *DNAH11* is found in approximately 6%. However, *DNAI2* is a much smaller gene and less costly to sequence than *DNAH11*; therefore, *DNAI2* was tested before *DNAH11*, with the goal of reducing overall sequencing costs. At the time of design, very little mutation frequency information was available for the remaining genes. As a result, these were ordered based on gene size, from smallest to largest, again to help reduce the costs of sequencing.

If a variant of unknown significance (VUS) was encountered, the ordering clinician would examine the VUS very

carefully and assess its pathogenicity before proceeding with the next gene for testing. A variety of criteria were used to predict the pathogenicity of the variant. A variant would be considered disease causing if it was found in conjunction with a mutation in the same gene and predicted to be deleterious through three protein prediction programs: SIFT, PolyPhen-2, and MutationTaster (36–38).

Results of genetic mutation testing were categorized into homozygote (two identical pathogenic mutations on a single PCD gene), compound heterozygote (two different pathogenic mutations on a single PCD gene), heterozygote (one pathogenic mutation on a PCD gene), or no mutations. Having two pathogenic mutations in a single PCD gene (either homozygote or heterozygote) was considered diagnostic for PCD.

This study was approved by the Research Ethics Boards of the Hospital for Sick Children and St Michael's Hospital.

Results

Clinical Phenotype

A total of 52 patients from 45 families with probable or definite PCD were enrolled in the study. Twenty-six were male (50%) and 26 were female (50%), with an age range of 12 months to 55 years. Thirty-eight families had only one member affected, and seven families had two individuals affected. The predominant ethnicities were white (50%) and Pakistani (29%), and parental consanguinity was present in 15 (33%) families. Situs solitus was present in 28 individuals (53%), whereas 21 (40%) had situs inversus, and 3 had situs ambiguous. The majority of patients experienced neonatal respiratory distress (85%), otitis media (75%), bronchiectasis (75%), and sinusitis (94%).

Ultrastructural Analysis of Cilia

Forty-nine patients had a ciliary biopsy at our center and were tested for ultrastructural defects by TEM using a qualitative and quantitative approach as previously described (5). Twenty-eight (57%) patients had clear ciliary defects, six (12%) had inconclusive findings, six (12%) samples had an inadequate number of ciliated cells for TEM analysis, nine (18%) samples had no apparent defect, and three individuals refused to have biopsy. Of the 28 individuals with confirmed ciliary defects, 13 had isolated ODA defects, 6 had IDA+CP defects, and 9 had combined ODA+IDA defects.

Mutation Profile

Patients with confirmed ciliary defects and positive genetic results. Eighteen patients (64%) from 15 families with confirmed ciliary defects were found to have at least one mutation (Table 1). In these patients, *DNAH5* was the most commonly mutated gene, found in eight families (53%), followed by *CCDC40* and *CCDC39* found in two families (13%) each, and *DNAI2* and *DNAAF2/KTU* in one family (6%) each. Consistent with previous reports, all patients with *DNAH5* or *DNAAF2/KTU* mutations had isolated ODA defects or combined ODA+IDA defects (19, 39, 40). Individual 23 was found to have a novel nonsense mutation, c.10508G>A (p.Trp3503*) and a second mutation, c.10616G>A (p.Arg3539His), which is similar to a previously documented mutation, p.Arg3539Cys in a family with PCD, which is believed to be involved in a critical microtubule binding site in DNAH5 (41), and we conclude this mutation to be disease causing. Individual 31 was found to have a novel nonsense mutation, c.9095C>G (p.Ser3032*) and a second missense mutation, c.11437C>T (p.Arg3138Trp), which is listed in dbSNP (rs140948494) with a minor allele frequency less than 0.1% (www.1000genomes.org). The Arg3138 amino acid is conserved among primate, chicken, frog, and fruit fly DNAH5 orthologs, and the Arg to Trp change is predicted to be deleterious (36–38). Given this allele's rarity and amino acid change, we conclude it to be disease causing.

Interestingly, family 127 had initial interpretation of the biopsy as apparently normal in individual 3 and inconclusive in individual 33. Given the molecular findings of two mutations in *DNAH5*, this prompted a review of both individuals (3, 33) and showed a classic ciliary defect with shortened stubby ODAs in both individuals.

Also consistent with the literature, patients with mutations in *CCDC39* and *CCDC40* had IDA+CP defects (patients 5, 7, 22, 27, 34), without any apparent ODA defects (26, 27). In four patients, only a single heterozygous pathogenic mutation in the *DNAH5* was detected. Individual 12 had a biopsy initially reported as inconclusive; however, when reviewed with the molecular findings, the patient was found to have a decreased number of ODA. In total, 17 novel mutations were found in the 15 families analyzed, along with 4 previously described mutations (39, 42).

Patients with nondiagnostic ciliary biopsies and positive genetic results. Nine (38%) of the patients with nondiagnostic biopsies had at least one expected pathogenic mutation (Table 2). Three patients, all with positive parental consanguinity, were found to have homozygous mutations, of whom patient 11 was found to have an undocumented missense variant (p.Leu2488Pro) in *DNAH5*. The Leu2488 amino acid is conserved among primate, chicken, frog, and fruit fly DNAH5 orthologs; the Leu to Pro change is predicted to be deleterious, and we conclude it to be disease causing (36–38). In addition, this patient also had an undocumented heterozygous missense variant (Arg121Cys; *see* Table E1 in the online supplement) in *DNAAF1/LRRC50* that is predicted to be benign.

Five patients from four different families were found to be compound heterozygotes. Two families, (128, 129) had affected individuals with a normal ciliary biopsy and two mutations in *DNAH11*, which is consistent with the literature (28). Of these, family 129 had one premature truncating mutation in *DNAH11* and a novel missense p.Ile4122Ser variant. Ile4122 is a well-conserved amino acid; this change is predicted to be deleterious, and we conclude this mutation to be disease causing (36–38). Similarly, individual 8 had one premature chain termination codon in *DNAH5* and a novel missense p.Ser3770Gly variant, which is also predicted to be pathogenic, and we conclude it to be disease causing (36–38). In total, seven novel mutations were found in the eight families, along with five previously reported mutations (28, 39, 43).

Patients with negative or inconclusive genetic results. Ten patients (40%) with confirmed ciliary defects did not have any detectable mutations in any of the genes tested (Table 3). Several rare and previously undocumented variants were identified in these patients (Table E1).

In 15 patients with nondiagnostic ciliary biopsies, no disease-causing genetic mutations were found. Two patients (14, 37) without a confirmatory biopsy had affected

Table 1. Patients with primary ciliary dyskinesia with confirmed ciliary defects and positive molecular genetic results

Family	Patient	Ethnicity	Sex	Age at Dx	Consanguineous	Situs Status	Neo RDE	Otitis Media	Bxsis	Sinusitis	Ciliary Defects	nNO	Gene	Base Changes	Predicted Effect	No. Genes Tested
Homozygous																
100	6	Pakistani	M	2 yr	Y	S	+	+	+	+	ODA+IDA	23.7	*DNAI2*	c.739C>T	p.Arg247*	5
101	13	Italian	F	9 yr	N	S	+	−	+	+	ODA+IDA	10.2	*DNAAF2*	c.31delG	p.Glu11Argfs*5	9
102	29	Afghanistani	F	14 yr	Y	I	+	+	+	+	ODA	13.1	*DNAH5*	c.9460_9461ins GTTTTTCT	p. Asp3154Glyfs*23	1
103	27	White	F	2 mo	N	I	+	+	+	+	IDA+CP	6.8	*CCDC39*	c.830_831delCA	p.Thr277Argfs*3	3
104	22	Pakistani	F	4 yr	Y	S	+	−	+	−	IDA+CP	5.6	*CCDC40*	c.1416delG	p.Ile473Phefs*2	4
104	34	Pakistani	F	3 yr	Y	S	+	+	+	+	IDA+CP	0.4	*CCDC40*	c.1416delG	p.Ile473Phefs*2	4
Compound heterozygous																
105	5	White	M	10 mo	N	I	+	−	−	+	IDA+CP	7.4	*CCDC40*	c.939+1G>C c.3097A>T	Splicing p.Lys1033*	4
106	7	White	M	3 yr	N	S	+	+	+	+	IDA+CP	6.5	*CCDC39*	c.830_831delCA c.1795C>T	p.Thr277Argfs*3 p.Arg599*	3
107	28	White	M	2.5 mo	N	I	+	−	−	+	ODA	ND	*DNAH5*	**c.10815delT** (39) c.11571-1G>A	**p.Pro3606Hisfs*23** Splicing	1
108	23	Pakistani	M	10 mo	N	S	+	−	−	+	ODA	9.7	*DNAH5*	c.10508G>A c.10616G>A	p.Trp3503* p.Arg3539His	1
109	31	White	M	7 yr	N	S	+	+	+	+	ODA	140.5	*DNAH5*	c.9095C>G c.11437C>T	p.Ser3032* p.Arg3813Trp	1
127	3	East Indian	F	16 yr	N	S	−	−	+	+	ODA	13.8	*DNAH5*	c.6086delG c.9286C>T	p.Gly2029Valfs*25 p.Arg3096*	1
127	33	East Indian	F	14 yr	N	S	+	−	+	+	ODA	17.7	*DNAH5*	c.6086delG c.9286C>T	p.Gly2029Valfs*25 p.Arg3096*	1
144	54	White	M	11 mo	N	I	+	+	−	+	ODA	18.9	*DNAH5*	**c.10384C>T** (42) **c.13338+5G>A** (39)	**p.Gln3462*** **Splicing**	1
Heterozygous																
111	4	White	M	4 yr	N	I	+	+	+	+	ODA	8.1	*DNAH5*	**c.13486C>T** (39)	**p.Arg4496***	12
112	32	Aboriginal	F	11 yr	N	A	+	+	−	+	ODA	25.6	*DNAH5*	c.9449delG	p.Gly3150Alafs*24	12
113	12	Portuguese	F	6 yr	N	A	+	+	+	+	ODA	22	*DNAH5*	c.4237C>T	p.Gln1413*	12
113	36	Portuguese	M	14 yr	N	S	+	+	+	+	ODA	8.9	*DNAH5*	c.4237C>T	p.Gln1413*	12

Definition of abbreviations: A = situs ambiguous; Bxsis = bronchiectasis; CP = central pair; F = female; I = situs inversus; IDA = inner dynein arms; M = male; N = no; Neo RDE = neonatal respiratory distress; nNo = nasal nitric oxide in nl/min; ODA = outer dynein arms; S = situs solitus; Y = yes.
Molecular genetic analysis was conducted on the following genes: *DNAH5*, *DNAI1*, *CCDC39*, *CCDC40*, *DNAI2*, *DNAH11*, *RSPH9*, *RSPH4A*, *DNAAF1*, *DNAAF2*, *TXNDC3*, *DNAL1*.
Documented mutations are indicated in boldface.

Table 2. Patients with probable primary ciliary dyskinesia with nondiagnostic biopsies and positive molecular genetic results

Family	Patient	Ethnicity	Sex	Age at Dx	Consanguineous	Situs Status	Neo RDE	Otitis Media	Bxsis	Sinusitis	Ciliary EM	nNO	Gene	Base Changes	Predicted Effect	# Genes Tested
Homozygous																
122	11	Pakistani	F	1.5 mo	Y	I	+	+	+	+	Not done	23.5	*DNAH5*	c.7463T>C	p.Leu2488Pro	12
123	47	Sri Lankan Tamil	M	53 yr	Y	I	–	+	+	+	Inadequate	3.1	*DNAI1*	c.1188delC	p.Tyr397Thrfs*5	2
124	52	Jordanian	F	1 mo	Y	I	–	+	+	+	Inadequate	7.2	*DNAH11*	**c.4348C>T** (28)	**p.Arg1450***	6
Compound heterozygous																
125	10	White	F	6 yr	N	I	+	+	–	+	Inadequate	5.6	*DNAH5*	c.3037_3040delAGCG	p.Val1014Leufs*20	1
														c.10815delT (39)	**p.Pro3606Hisfs*23**	
126	8	White	M	0 mo	N	I	+	+	–	+	Inconclusive	9.7	*DNAH5*	**c.13458dupT** (39)	**p.Asn4487***	12
														c.11308A>G	p.Ser3770Gly	
128	41	Pakistani	F	2 yr	N	I	+	+	–	+	Normal	22	*DNAH11*	**c.2569C>T** (28)	**p.Arg857***	6
														c.4471_4473delCTAinsAT	p.Leu1491Ilefs*11	
129	42	Pakistani	M	10 yr	Y	A	+	+	–	+	Normal	7.3	*DNAH11*	c.12365T>G	p.Ile4122Ser	12
														c.12888T>A	p.Cys4296*	
129	43	Pakistani	F	3 yr	Y	I	–	–	–	–	Normal	5.1	*DNAH11*	c.12365T>G	p.Ile4122Ser	12
														c.12888T>A	p.Cys4296*	
Heterozygous																
130	40	White	M	5 yr	N	S	+	+	+	+	Normal	9.8	*DNAH11*	**c.8719C>T** (43)	**p.Arg2907***	12

Definition of abbreviations: A = situs ambiguous; Bxsis = bronchiectasis; EM = electron microscopy; F = female; I = situs inversus; inadequate = inadequate ciliated cells to analyze; inconclusive = adequate sample, inconclusive transmission electron microscopy; M = male; N = no; ND = not done; Neo RDE = neonatal respiratory distress; nNo = nasal nitric oxide in nl/min; S = situs solitus; Y = yes.

Molecular genetic analysis was conducted on the following genes: *DNAH5*, *DNAI1*, *CCDC39*, *CCDC40*, *DNAI2*, *DNAH11*, *RSPH9*, *RSPH4A*, *DNAAF1*, *DNAAF2*, *TXNDC3*, *DNAL1*.

Documented mutations are indicated in boldface.

siblings (patients 26 and 35, respectively), both with combined ODA+IDA defects. Thus, the suspicion of PCD in these two patients is very high, even without confirmation by ciliary biopsy or molecular testing. Of the remaining 13 patients, 5 had normal biopsies, 5 were inconclusive, 2 were inadequate, and 1 refused biopsy. Based on their constellation of clinical findings and low nasal nitric oxide, these individuals were considered to be probable PCD, although not confirmed on TEM or molecular testing.

Diagnostic Yield

The diagnostic yield of ciliary biopsy alone was 57% (28 of 49) (Tables 4 and 5). Although 27 of 52 (52%) individuals had at least one allele harboring mutation in the 12 PCD genes tested, only 22 of 52 (42%) had two pathogenic mutations to confirm the genetic diagnosis of PCD. In total, 36 individuals were diagnosed with PCD. Fourteen were diagnosed through biopsy alone, 8 with positive molecular results, and 14 had both diagnostic biopsies and two disease-causing mutations. Overall, in this population, adding clinically available molecular genetic testing increased the diagnostic sensitivity to 69% (36 of 52) compared with 57% based on biopsy alone.

Discussion

The diagnosis of PCD is challenging and currently relies on clinical features and ciliary analysis. This is, to our knowledge, the first study to assess and compare the diagnostic usefulness of molecular genetic analysis in a clinical setting. Historically, patients have been diagnosed with PCD based on TEM of ciliary tissue. However, the diagnostic yield of such studies is reported between 20 and 39% (4, 5), and many individuals refuse to have such an invasive procedure. In our study, a slightly higher diagnostic yield of 57% was observed, which may be a reflection of a different patient selection process for biopsy in our respective centers. In addition, inconclusive biopsies were observed in six individuals (12% of biopsy samples), which is consistent with the 11 to 22% reported previously (4, 5). The individuals with inconclusive and inadequate biopsies were offered repeat biopsy; however, the majority refused. Two individuals (21, 50) who did agree to repeat nasal scraping had

Table 3. Patients with primary ciliary dyskinesia with inconclusive molecular genetic results

Family	Patient	Ethnicity	Sex	Age at Dx	Consanguineous	Situs Status	Neo RDE	Otitis Media	Bxsis	Sinusitis	Ciliary EM	nNO
Confirmed ciliary defect												
110	15	Pakistani	F	8 yr	Y	S	+	+	+	+	ODA+IDA	10.8
114	9	White	M	4 yr	N	S	+	+	+	+	ODA	13.4
115	26	East Indian	M	9 yr	N	S	+	+	+	+	ODA+IDA	5.8
116	16	Pakistani	F	15 yr	Y	S	+	–	+	+	ODA	28.9
117	17	Somali	M	14 yr	Y	I	+	–	–	+	ODA+IDA	107.2
117	19	Somali	M	1 yr	Y	I	+	+	–	+	ODA+IDA	8.8
118	35	Pakistani	F	2 mo	Y	S	+	+	+	+	ODA+IDA	23.4
119	18	White	F	11 yr	N	S	+	+	+	+	ODA+IDA	1.0
120	24	White	M	2 mo	N	I	+	+	–	+	ODA+IDA	11.2
121	30	Portuguese	M	9 yr	N	S	+	+	+	+	IDA+CP	15.3
Unconfirmed ciliary defect												
132	21	White	M	6 yr	N	I	+	+	+	+	Normal	4.9
133	25	White	M	5 yr	N	S	+	+	+	+	Normal	13.8
134	38	Hispanic	M	15 yr	N	S	+	+	+	–	Normal	17
136	44	Portuguese	M	8 yr	N	S	+	+	+	+	Normal	20.1
138	46	White	F	35 yr	N	S	+	+	+	+	Normal	53.2
131	20	White	M	2 yr	N	I	+	+	+	+	Inconclusive	99.2
139	48	Pakistani	F	33 yr	Y	S	+	+	+	+	Inconclusive	56.2
141	50	Pakistani	F	5 yr	N	I	+	+	+	+	Inconclusive	17.1
142	51	Sri Lankan Tamil	F	18 yr	N	S	+	–	+	+	Inconclusive	14.5
143	53	White	F	20 yr	Y	S	+	–	+	+	Inconclusive	18.4
135	39	Pakistani	F	12 yr	Y	S	+	+	+	+	Inadequate	22.1
137	45	Hispanic	F	30 yr	N	S	+	–	+	+	Inadequate	17.1
115	14	East Indian	M	7 yr	N	S	+	+	+	+	Inadequate	37.5
118	37	Pakistani	M	1 yr	Y	I	+	+	+	+	Not done	36.5
140	49	Portuguese	F	18 yr	Y	I	?	–	+	+	Not done	6.9

Definition of abbreviations: A = situs ambiguous; Bxsis = bronchiectasis; CP = central pair; EM = electron microscopy; F = female; I = situs inversus; IDA = inner dynein arms; inadequate = inadequate ciliated cells to analyze; inconclusive = adequate sample, inconclusive transmission electron microscopy; M = male; N = no; ND = not done; Neo RDE = neonatal respiratory distress; nNo = nasal nitric oxide in nl/min; ODA = outer dynein arms; S = situs solitus; Y = yes.
Molecular genetic analysis was conducted on the following genes: *DNAH5*, *DNAI1*, *CCDC39*, *CCDC40*, *DNAI2*, *DNAH11*, *RSPH9*, *RSPH4A*, *DNAAF1*, *DNAAF2*, *TXNDC3*, *DNAL1*.

inconclusive biopsies on their repeat samples. This is a reflection of the limitations of the overall diagnostic usefulness of biopsy outside of the setting of a clinical trial.

The diagnosis of PCD based on ciliary biopsy alone is further complicated as individuals with PCD and *DNAH11*, *HYDIN*, *CCDC164*, and *CCDC65* mutations have normal-appearing ciliary ultrastructure by TEM (28–31). Furthermore, some ultrastructural defects, such as IDA and radial spoke defects, were found to be false positives, having normal TEM and beat pattern analysis on repeat biopsy (44).

Genetic testing overcomes technical limitations of ciliary analysis and has been believed to increase the diagnostic yield in patients with suspected PCD; however, despite its convenience and availability, it is not used routinely in clinical practice (3). Of the 52 patients who underwent genetic testing using the PCD panel of 12 genes, 22 individuals were found to have two mutations in known genes associated with PCD. Eight of these individuals had nondiagnostic biopsies (not done, inadequate, inconclusive, or normal), providing an additional diagnostic yield of 12% compared with biopsy alone. Together, genetic testing in conjunction with ciliary biopsy provides an overall diagnostic rate of 69% for patients suspected of having PCD. Furthermore, a molecular genetic result offers carrier testing, prenatal testing, and genetic counseling, which are not possible with a pathologic diagnosis.

Ultrastructural analysis of cilia is challenging, and TEM is prone to false-positive and -negative results (4, 5). Our studies used standard operating protocols, and independent reviewers examined samples. Unfortunately, such procedures are not routine in most nonacademic settings, particularly in nonpediatric hospitals, and depending on the electron microscopist's familiarity with PCD abnormalities, including sample preparation and analysis, results may be variable. Here, we demonstrate that the molecular findings in patients can result in review of the cilia ultrastructure and can contribute to the pathologic interpretation of the ciliary ultrastructure.

In addition, we document 24 previously unreported genetic variants in various genes (Table E2), of which five were classified as disease causing based on their effects on

Table 4. Categorization of patients with primary ciliary dyskinesia based on diagnostic modality

	Diagnostic Ciliary Biopsy	Noncontributory Ciliary Biopsy†	Totals
Diagnostic molecular genetics*	14	8	22
Nondiagnostic molecular genetics	14	16	30
Total	28	24	52

Molecular genetic analysis was conducted on the following genes: *DNAH5, DNAI1, CCDC39, CCDC40, DNAI2, DNAH11, RSPH9, RSPH4A, DNAAF1, DNAAF2, TXNDC3, DNAL1*.
*Includes biopsies that were normal, inconclusive, inadequate, or not done.
†Two pathogenic mutations in a single primary ciliary dyskinesia gene.

protein structure and the presence of another pathogenic mutation. A total of 42% of our patients were found to have two mutations in a PCD gene, which is similar to that reported in the literature (1).

Despite providing additional diagnostic information on patients with two clearly pathogenic mutations, molecular testing conducted here may also require careful interpretation and potentially further testing. For example, in those individuals with only one mutation detected, it is difficult to definitively conclude pathogenicity. Considering all five were chain termination mutations, it is likely these are the genes responsible for PCD in these patients. Furthermore, the biopsy results are congruent with the molecular results, which can help guide further molecular testing options. Because all coding exons and intron/exon boundaries were sequenced, additional studies, such as deletion/duplication testing and mRNA sequencing, will be necessary to identify a possible second pathogenic allele and to confirm that these mutations are causing PCD in these patients. *In vitro* ciliary beat frequency analysis would also be helpful in determining the pathogenicity of these cases. Alternatively, in some cases, biallelic mutations in another gene that is not tested may be responsible for the PCD phenotype.

Furthermore, the genetics of PCD are not fully elucidated, as highlighted in individual 50, who had one previously documented predicted pathogenic VUS (Table E1), c.520G>A in *DNAI1* (40), and a common *TXNDC3* intronic variant, c.271–27 C>T, previously documented in a family with PCD (6). A second mutation was not found in these two genes or in the rest of the 10 PCD genes offered on the panel. We can speculate that this individual had two mutations on different genes; however, digenic inheritance has not previously been reported in PCD, and it is difficult to ascertain if that is the case for this patient. Another possibility is that this individual carries a large deletion as the second mutated allele or biallelic mutations in another locus involved in PCD.

As more than one-third of individuals with well-characterized PCD do not have a mutation in associated PCD loci, molecular genetic analysis may not provide a diagnosis in all cases. In 25 individuals in our study, genetic testing was not helpful, in that testing 12 known genes resulted in finding no mutations. This is a significant portion (48%) of the population tested. These individuals may possess a mutation not detectable using the current molecular assay and may benefit from testing the 12 other PCD-associated genes or gene-discovery experiments. Due to the lag of newly discovered PCD genes being clinically available, our study may be underpowered in terms of gene coverage. We expect with the ongoing discovery of new PCD loci that the proportion of individuals with unknown PCD loci will decrease, increasing the diagnostic contribution of molecular genetic analysis.

There are a variety of limitations of this current study. For a recessively inherited disease such as PCD, a clear genetic diagnosis requires two pathogenic mutations on opposite chromosomes. However, because parents have not been tested, we cannot rule out the possibility that both mutations are *in cis* for compound heterozygotes or that a large intergenic deletion is present in homozygotes (Table 5). Given the clinical history of the patients in this study, and the fact that nearly all heterozygous mutations are predicted strong loss of function (i.e., frameshift, nonsense, splicing), we presume patients with both mutations on the same chromosome will be a minority. Likewise, we expect individuals with a large deletion covering a pathogenic mutation to be a minority, because all but two (13, 27) are reported to be from consanguineous parents. Nevertheless, parental studies are currently underway.

Here, we describe a multiethnic cohort of 52 patients with a suspected diagnosis of PCD based on clinical history and diagnostic investigations who subsequently underwent molecular genetic analysis. Importantly, eight individuals, whose

Table 5. Categorization of genetic mutation zygosity based on ciliary biopsy results in 52 patients with primary ciliary dyskinesia

	Homozygote	Compound Heterozygote	Heterozygote	No Mutations
Diagnostic ciliary biopsy	6	8	4	10
Noncontributory ciliary biopsy*	3	5	1	15
Total	9	13	5	25

Molecular genetic analysis was conducted on the following genes: *DNAH5, DNAI1, CCDC39, CCDC40, DNAI2, DNAH11, RSPH9, RSPH4A, DNAAF1, DNAAF2, TXNDC3, DNAL1*.
*Includes biopsies that were normal, inconclusive, inadequate, or not done.

diagnosis was not obtained through a biopsy of cilia, were found to have two mutations in associated PCD genes, increasing the combined diagnostic yield with genetic testing to 69%. Based on these results, we suggest genetic testing be conducted on all individuals with a suspected diagnosis of PCD in conjunction with ciliary biopsy to maximize the diagnostic accuracy. As not all the genes associated with PCD are known, molecular genetic analysis should not be considered the sole diagnostic modality. However, genetic testing can be used as a convenient initial test for individuals in whom the clinical suspicion for PCD is very high and access to ciliary biopsy is limited. In addition, positive molecular findings have the added advantage of giving the family useful genetic information for future prenatal diagnosis and carrier testing studies. Importantly, the diagnostic yield of molecular analysis for PCD is predicted to increase greatly as new PCD genes are discovered and added to clinical sequencing panels. As the diagnostic yield increases and the cost of DNA sequencing continues to drop, molecular analysis for highly heterogeneous genetic diseases such as PCD is expected to become an invaluable and indispensable tool for clinicians. ■

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