DNAH5 Mutations Are a Common Cause of Primary Ciliary Dyskinesia with Outer Dynein Arm Defects


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Rationale: Primary ciliary dyskinesia (PCD) is characterized by recurrent airway infections and randomization of left–right body asymmetry. To date, autosomal recessive mutations have only been identified in a small number of patients involving DNAI1 and DNAH5, which encode outer dynein arm components.

Methods: We screened 109 white PCD families originating from Europe and North America for presence of DNAH5 mutations by haplotype analyses and/or sequencing.

Results: Haplotype analyses excluded linkage in 26 families. In 30 PCD families, we identified 33 novel (12 nonsense, 8 frameshift, 5 splicing, and 8 missense mutations) and two known DNAH5 mutations. We observed clustering of mutations within five exons harboring 27 mutant alleles (52%) of the 52 detected mutant alleles. Interestingly, 6 (32%) of 19 PCD families with DNAH5 mutations from North America carry the novel founder mutation 10815delT. Electron microscopic analyses in 22 patients with PCD with mutations invariably detected outer dynein arm ciliary defects. High-resolution immunofluorescence imaging of respiratory epithelial cells from eight patients with DNAH5 mutations showed mislocalization of mutant DNAH5 and accumulation at the microtubule organizing centers. Mutant DNAH5 was absent throughout the ciliary axoneme in seven patients and remained detectable in the proximal ciliary axoneme in one patient carrying compound heterozygous splicing mutations at the 3′-end (IVS75-2A>T, IVS76+5G>A). In a preselected subpopulation with documented outer dynein arm defects (n = 47), DNAH5 mutations were identified in 53% of patients.

Conclusions: DNAH5 is frequently mutated in patients with PCD exhibiting outer dynein arm defects and mutations cluster in five exons.

Keywords: cilia; DNAH5; outer dynein arm; primary ciliary dyskinesia

Primary ciliary dyskinesia (PCD; Mendelian Inheritance in Man [MIM] 242650) is estimated to affect 1 in 10,000 to 20,000 individuals (1, 2). The complex PCD phenotype involving various organ systems is explained by dysfunction of motile cilia and flagella, respectively. Recurrent respiratory infections are caused by defective mucociliary clearance due to immotile or dysmotile respiratory cilia (3), resulting in chronic inflammation and bronchiectasis. Dysfunction of monocilia at the embryonic node is associated with randomization of left–right body asymmetry (4), and half of the patients with PCD exhibit situs inversus. PCD plus situs inversus is also referred to as Kartagener’s syndrome (MIM 244400). Reduced fertility is often observed in male patients with PCD (5, 6). The inconclusive initial clinical presentation, particularly in children without situs inversus, often delays diagnosis and may cause underestimation of the disease prevalence (7).

Respiratory cilia activity is generated by the action of dynein arm complexes attached to the peripheral axonemal A-microtubules (8–10). In PCD, various ultrastructural defects of the ciliary axoneme have been identified. The most frequent abnormalities comprise the absence of some or all outer dynein arms (ODAs) (3); other defects include inner dynein arm (IDA) defects, microtubule transpositions, and radial spoke defects (11).

PCD represents a heterogeneous group of genetic disorders. In most cases, inheritance is autosomal recessive but other modes of transmission have also been observed. DNAI1, the human ortholog of the Chlamydomonas reinhardtii gene IC78, encodes an intermediate chain of the ODA (12). To date, recessive DNAI1 mutations have been detected in 6 of 47 studied PCD families (13, 14), and approximately 50% of reported mutant alleles comprise the splicing mutation 219+3insT. Occasionally, mutations in the RPGR gene located on the X chromosome, which is responsible for 20% of all retinitis pigmentosa cases, have been found in males with retinal degeneration and PCD (15–17).

Applying a homozygosity mapping strategy, we previously localized a PCD locus to chromosome 5p (18). Using a candidate gene approach, we identified DNAH5, which is composed of 79 exons with one alternative first exon. DNAH5 encodes a heavy chain of the ODA and is the human homolog of the dynein γ-heavy chain of C. reinhardtii. Mutational analysis in 25 PCD families in which haplotype analyses were consistent with linkage to the DNAH5 locus detected recessive mutations in eight PCD families (19). Here, we analyzed additional 109 PCD families with various ultrastructural phenotypes for mutations in DNAH5. Considering the previous report of extensive genetic heterogeneity in PCD (20), we unexpectedly found that DNAH5 mutations are responsible for PCD in 28% of 109 analyzed PCD families. Interestingly, half of PCD families with ODA defects harbored DNAH5 mutations.
METHODS

Patients
We collected blood samples from 134 affected PCD subjects and family members belonging to 109 unrelated nuclear families. Ultrastructural analyses revealed an ODA defect in 47 PCD families, whereas only nine families exhibited an IDA defect (see the online supplement Figure E2). Twenty-one families had parental consanguinity and diagnosis of PCD was based on established criteria (1). Pedigrees of PCD families were compatible with autosomal recessive inheritance. The geographic origin was Europe (Germany, 46; United Kingdom, 10; Switzerland, 10; Hungary, 8; Belgium, 1; The Netherlands, 2; Austria, 2; and Sweden, 1) and North America, 29. To establish representative prevalence data, we added the 25 PCD families analyzed in our previous study (19; see Figures E3 and E4). In addition, 60 unrelated North American patients were screened for the founder mutation 10815delT (32 had ODA defects, 3 had IDA defects, 2 had central pair defects, 9 had normal dynein arms). Informed consent was obtained using protocols approved by the Institutional Ethics Review Board at the University of Freiburg and collaborating institutions.

Haplotype Analysis
Haplotype analysis was performed in consanguineous families and families with at least two affected individuals. Two highly polymorphic intragenic microsatellite markers (MIC-DNAH5-1; MIC-DNAH5-3) were tested as described previously (19). Using sequence information of 60 single-nucleotide polymorphisms, we deduced haplotypes based on genotypes of patients with homozygous mutations (F373, UNCB, UNC47, UNC83) and the assumption of existing founder haplotypes in patients with PCD originating from different families.

Mutational Analysis
Genomic DNA was isolated by standard methods directly from blood samples or from lymphocyte cultures after Epstein-Barr virus transformation. Amplification of 80 genomic fragments comprising all 79 exons and one alternative first exon of DNAH5 was performed as described previously (19). Sequence data were evaluated using the CodonCode software (CodonCode Corporation, Dedham, MA). To aid identification of frequent polymorphisms, we sequenced samples from 10 healthy control subjects. All identified mutations were confirmed by repeated sequencing. We screened 140 control chromosomes originating from healthy white individuals for the presence of missense and facultative splicing mutations. Segregation analyses and screening of control subjects were performed by restriction analysis, denaturing high-performance liquid chromatography, or sequencing.

cDNA Analysis of the 1730G→C Mutation
Total RNA was isolated by the TRIzol method or RNeasy Mini Kit (Qiagen, Valencia, CA) from respiratory epithelial cells obtained by nasal brush biopsies. First-strand cDNA synthesis was performed with a SuperScriptII RNase kit (Invitrogen, Carlsbad, CA). As a control, reverse transcriptase was omitted in a parallel reaction. To test whether the splice donor site of exon 13 is mutated, a cDNA fragment corresponding to exons 12–14 was amplified by polymerase chain reaction (PCR; 52°C annealing temperature) with primers spanning the junctions of exons 11/12 and 14–15 (F: 5′-TAA ATA CCA GGG CAT TGT GG-3′; R: 5′- TTC TTC TCC AAT TTG CCG AAG C-3′), respectively. Products were subjected to second-round amplification, cloned, and subsequently sequenced (see Figure E5).

High-Speed Video Analysis of Cilia Beating
Ciliary beat frequency and beating pattern were assessed with the Sisson Ammons Video Analysis (SAVA) system as described previously (21). We examined nasal brush biopsies from five patients with PCD (OP40, OP51, F718, OP54, OP31) in which novel DNAH5 mutations were detected.

Immunofluorescence Studies of Respiratory Epithelial Cells
Respiratory epithelial cells from patients OP9, OP31, OP40, OP51, OP54, F718, UNC29, and UNC35 were obtained by transnasal brush biopsy and processed for high-resolution immunofluorescence imaging as described previously (21).

RESULTS

Mutational Analysis of DNAH5
We have previously shown that DNAH5 mutations cause PCD and randomization of left–right asymmetry (19) (see Figure E3). To determine the frequency of DNAH5 mutations in PCD, we screened an additional large international cohort of 109 PCD families (see Figure E2). In 26 PCD families, haplotype analyses were not consistent with linkage to the DNAH5 locus, indicating that DNAH5 mutations most likely are not responsible for the disease phenotype in these families. Sequencing of all coding exons, including the exon/intron boundaries in the remaining 83 families, revealed 33 novel DNAH5 mutations in patients with PCD from 30 families or 34 affected patients (Figure 1). In addition, the two previously reported frameshift mutations, 5563_5564insA and 8440-8447delGAAAACAAA, were also identified in three and one patients, respectively (19). In only eight families, one mutant allele was detected and the second mutant allele remained unidentified (Table 1). The observed mutational changes fell into two major groups; truncating (n = 27; 77%) and missense mutations (n = 8; 23%). The 33 novel mutations included 12 nonsense mutations, eight frameshift mutations, three obligatory and two facultative splicing mutations (1730G>C; IVS76+5G>A), as well as eight missense mutations.

The missense and facultative splicing mutations were absent in 140 control chromosomes, and cosegregation of the mutations with disease phenotypes was confirmed (Table 1). For facultative splicing mutation (1730G>C), aberrant splicing was confirmed by reverse transcriptase–PCR analysis (see below). In the IVS76+5G>A mutation, a conserved (80%) base pair is affected, which predicts aberrant splicing. We considered the IVS76+5G>A exchange to represent a mutation, because this DNA variant was found twice in the PCD population but was absent in the control population. In addition, cosegregation within the two PCD families carrying the IVS76+5G>A was noted and deduced haplotype analysis (see below) was consistent with a founder mutation.

The eight identified missense mutations affect amino acid residues that have been evolutionarily conserved in the mouse (AF466704) and/or C. reinhardtii DNAH5 orthologs, respectively. Two of these mutations are located within known functional domains of DNAH5 (Figure 1). The mutation W3409S is located in the microtubule binding site and S2264N resides in the P2-loop domain of the protein.

We observed clustering of mutations within five exons harboring 27 mutant alleles (52%) of the 52 detected mutant alleles. Clinical findings of PCD patients with DNAH5 mutations are depicted in Table E2. No genotype–phenotype correlations have been identified.

The 1730G>C Mutation Results in Abnormal Splicing
In Patient UNC75 II1, we detected the heterozygous 1730G>C mutation, which affects an 80% conserved base pair of the splice donor site of exon 13. To test whether this mutation indeed affects splicing, we performed reverse transcriptase–PCR, which showed two fragments corresponding to the wild-type (535 bp) and a variant (449 bp) allele, consistent with heterozygosity (see Figure E5). Cloning and subsequent sequencing of the PCR products revealed that the 449-bp product fuses exon 12 out of frame to exon 14 and therefore lacks exon 13, whereas the 535-bp product coincides with the wild-type fragment. We conclude that the mutant splice site cannot be recognized by the splicing machinery, leading to skipping of exon 13, which predicts premature termination of translation because of out-of-frame fusion.
mutations were identified in 53% of patients. DNAH5 in nine individuals with isolated IDA defects, we did not find encoding an ODA heavy chain most likely affect only DNAH5 and/or high-resolution immunofluorescence microscopy (n documented ODA defects demonstrated by electron microscopy. In the preselected subpopulation of patients with PCD with assembly of ODA complexes, but might not affect IDA assembly. Mutations that were detected in several unrelated families are marked by an asterisk (> 4 PCD families) and dagger (2 PCD families). Mutations that were identified in our previous study are indicated by gray boxes (19). Novel mutations are depicted in red.

DNAH5 Mutations Cause PCD with ODA Defects
All patients carrying DNAH5 mutations that were studied by transmission electron microscopy (n = 22) exhibited ODA defects. We have previously shown that at least two distinct ODA complexes are found within human respiratory cilia and that both contain DNAH5 and exhibit a distinct regional distribution along the ciliary axoneme (21). In addition, we could demonstrate in three patients carrying homozygous DNAH5 mutations (5563_5564insA, 8440-8447delGAACCAA, and IVS74-1G>C) that the mutated DNAH5 proteins were expressed but were absent from the entire ciliary axoneme, and instead accumulated at the ciliary base. This finding indicates that these DNAH5 mutations affect assembly of both ODA types. Here, we studied an additional eight individuals with DNAH5 mutations for DNAH5 localization in respiratory cells that were obtained by nasal brushing biopsy. In all cases, we found expression of mutant DNAH5. In seven individuals, we found a complete absence of mutant DNAH5 from the ciliary axoneme and accumulation at the ciliary base and within the cytoplasm (Figure 2B), as reported previously (21). However, one patient (F718 II1) carrying compound heterozygous mutations, which affect splicing at the 3'-end of the gene (IVS 75 and 76), showed absence of DNAH5 only from the distal part of the ciliary axonemes (Figure 2C) and remaining DNAH5 staining in the proximal part, indicating that assembly of one type of ODA is at least partially preserved. We previously observed this pattern of aberrant DNAH5 staining only in patients with DNAI1 mutations (21). Interestingly, in nine individuals with isolated IDA defects, we did not find DNAH5 mutations by sequence analysis. Thus, mutations of DNAH5 encoding an ODA heavy chain most likely affect only assembly of ODA complexes, but might not affect IDA assembly. In the preselected subpopulation of patients with PCD with documented ODA defects demonstrated by electron microscopy and/or high-resolution immunofluorescence microscopy (n = 47), DNAH5 mutations were identified in 53% of patients.

We have previously shown that cilia with ODA defects are immotile or only show flickering movements (21). We analyzed patients with DNAH5 mutations by high-speed videomicroscopy (n = 5). Two of these patients (OP54, OP51) had completely immotile cilia in all examined respiratory epithelial cells. In three patients (OP31, OP40, F718), most respiratory cilia were immotile; however, some cilia showed severely altered motility with stiff movements and low amplitudes.

DNAH5 Founder Mutations
The mutations 10815delT, 5563_5564insA, 13458_13459insT, IVS76+5G>A, 8528T>C, and 8440–8447delGAACCAA were found in seven, three, four, two, and one families, respectively. Two of these mutations (5563_5564insA, 8440-8447delGAACCAA) were reported previously (19). To address the question of whether these mutations were derived from a common ancestor (founder) or occurred independently, we took advantage of the high number of single-nucleotide polymorphisms present in the 250-kb DNAH5 locus to deduce the haplotypes associated with the identified mutations. Because of the large dataset, results representative (n = 39) of 60 single-nucleotide polymorphisms are depicted in Figure E1. The haplotype analysis was facilitated, because some affected individuals carried homozygous mutations (5563_5564insA, 10815delT, 13458_13459insT). Analysis of the deduced haplotypes indicates that the mutations 10815delT (UNCB, UNC47, UNC35, UNC74, UNC29, UNC102, OP19), 13458_13459insT (UNCB, UNC47, UNC76, F718), and 8528T>C (UNC35, UNC48) are founder mutations (see Figure E1). Interestingly, for the mutations 5563_5564insA and 13458_13459insT, we could show that the mutational events most likely occurred independently in some of the families, whereas in other families, they are consistent with founder mutations (see Figure E1). Consistent with these findings, the 5563_5564insA mutation was identified in patients with PCD from Europe (OP31 III1) and North America (UNC72 and UNC76) who share...
the same haplotype. In contrast, their haplotype differs from the haplotype of an Arab patient with PCD (F373), who carries the S563_5564insA mutation reported previously (19). Similarly, we found three North American patients with PCD (UNC45, UNC74, and UNC83) with identical deduced haplotypes carrying the 13458_13459insT mutation and one patient with PCD from Europe (OP40 II1) with a different deduced haplotype. The 10815delT founder mutation was found in six United States’ families that have been thus far analyzed for the frequency of mutations in all 134 apparently unrelated PCD families and detected 33 distinct novel mutations and two mutations reported previously (Table 1). Similarly, we found three North American patients with PCD (UNC45, UNC74, and UNC83) with identical deduced haplotypes carrying the 13458_13459insT mutation and one patient with PCD from Europe (OP40 II1) with a different deduced haplotype. The 10815delT founder mutation was found in six United States’ families and detected 33 distinct novel mutations and two mutations reported previously (Table 1).

**TABLE 1. DNAHS MUTATIONS IN PRIMARY CILIARY DYSKINESIA**

<table>
<thead>
<tr>
<th>Family</th>
<th>Patient</th>
<th>Origin</th>
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<th>DNA Change</th>
<th>Protein Change</th>
<th>ODA Defect</th>
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*Definition of abbreviations: EM = electron microscopy; IF = immunofluorescence; M = maternal; mut. = mutation; n.a. = not available; n.d. = not determined; ODA = outer dynein arm; P = paternal.*

The most frequent ultrastructural defects in PCD affect the composition of dynein arms, which are responsible for cilia and sperm tail movement (11, 22–24). Linkage studies suggest extensive genetic heterogeneity in PCD (20). So far, only two genes, DNAH1 and DNAH5, have been clearly linked to autosomal recessive forms of PCD (12, 19). DNAH11 mutations possibly also cause PCD (25). All three genes encode for components of ODA complexes.

This study determined the frequency, distribution, and the phenotype associated with DNAH5 mutations. DNAH5 is a large gene comprising 79 exons and one alternative first exon and encodes a heavy chain of the ODA. We have recently shown that recessive DNAH5 mutations are responsible for PCD characterized by ODA defects (19). The murine ortholog Mdnah5 (alias Dnalic5) is expressed in the node during early embryonic development, in the respiratory tract and in testes, which is consistent with disease manifestations in PCD (19, 26). In this current study, we report analysis of an additional PCD cohort comprising 109 PCD families and detected 33 distinct novel mutations and two mutations reported previously (Table 1). Mutations were scattered along the entire coding region (Figure 1).
Most of the identified mutations were nonsense mutations, but frameshift, splicing, and missense mutations were also found. The most common DNAH5 mutations clustered within only five exons, 34, 50, 63, 76, and 77, which harbor DNAH5 mutations in 53% of a total of 134 analyzed PCD families (see Table E1 and Figure E4). The increased frequency of mutant alleles in only 5 of the 80 known coding exons of DNAH5 might help to develop a fast and cost-effective genetic assay for genetic diagnosis in the future. Six mutations (5563_5564insA, 10815delT, 13458_13459insT, 8440-8447delGAACAA, 8528T, and IVS76+5G>A) were identified in more than one unrelated PCD family. Three of the identified DNAH5 mutations (5563_5564insA, 10815delT, and 13458_13459insT) were found in four, seven, and four families, respectively. Based on the large number of single-nucleotide polymorphisms within the DNAH5 locus, we were able to analyze whether these sequence variants occurred because of independent mutational processes or are related to ancestral founders. These analyses indicate that 10815delT, 8528T>C, and IVS76+5G>A are founder mutations (Figure E1). For two of the other mutations (5563_5564insA and 13458_13459insT), we found a more complex situation, where, in some PCD families, the mutations probably occurred independent from each other and, in others, deduplicated haplotypes suggest a common ancestor (Figure E1). Importantly, we could demonstrate that the 10815delT mutation is especially prevalent in the white PCD population in the United States. We confirmed that genetic screening for this mutation is notably useful in United States’ PCD families and identified additional four PCD families in a cohort of 60 analyzed PCD families in the United States.

Previously, we studied the axonemal ultrastructure in several patients with PCD carrying DNAH5 mutations. All patients exhibited deficiency of ODAs (19, 26), which is consistent with DNAH5 constituting one component of the large multimeric ODA complexes. Here, evaluation of electron microscopic data from a large cohort of patients (n = 109) with DNAH5 mutations (n = 22) confirmed ultrastructural ODA defects in all cases, including patients carrying missense mutations. Interestingly, we did not find DNAH5 mutations in patients without ODA defects by electron microscopy. Importantly, patients with PCD with isolated IDA defects did not harbor DNAH5 mutations. On the basis of these observations, we conclude that DNAH5 mutations regularly disturb the correct assembly of ODA complexes.

To gain more insight into the molecular pathology of PCD caused by DNAH5 mutations, we analyzed respiratory cells obtained by nasal brushing biopsy from eight patients with PCD with recessive DNAH5 mutations. We have previously shown that human respiratory cilia contain at least two ODA types, which differ in their content of dynein heavy chains and which show differences concerning their regional distribution along the ciliary axoneme: type 1 (DNAH9-negative and DNAH5-positive; proximal ciliary axoneme) and type 2 (DNAH9- and DNAH5-positive; distal ciliary axoneme) (21). In the study presented here, we show by high-resolution immunofluorescence imaging that, regardless of the mutational background, mutant DNAH5 proteins are expressed in respiratory epithelial cells from patients with PCD, but are not appropriately localized along the axonemal shaft (Figure 2). Consistent with the available ultrastructural data exhibiting ODA deficiency, we identified in all eight analyzed patients with PCD with DNAH5 mutations mislocalization of mutant DNAH5 with accumulation at the microtubule organizing centers. Because all mutations result in mutant DNAH5 with preservation of the N-terminal third of the protein, we assume that this region contains a domain responsible for correct targeting of DNAH5 to the microtubule organizing center. The increased frequency of mutant alleles in only 5 of the 80 known coding exons of DNAH5 might help to develop a fast and cost-effective genetic assay for genetic diagnosis in the future.
centers. In all but one patient with PCD with DNAH5 mutations, we found complete absence of mutant DNAH5 from the respiratory ciliary axonemes, as reported previously in three patients with PCD with DNAH5 mutations (21). In these cases, mutations affect assembly of both ODA complexes that contain DNAH5 (Figure 2B). In contrast, in one patient (F718), we found that mutant DNAH5 was absent from the distal ciliary axoneme, but was preserved within the proximal ciliary axoneme (Figure 2C).

This staining pattern clearly demonstrates that, in this patient, mutant DNAH5 can be correctly targeted to the site of proximally located ODA complexes. F718 carries compound heterozygous mutations located at the 3’-end of DNAH5 (IVS 75 and 76), which both predict aberrant splicing. This indicates that the C-terminal region of DNAH5 is important for assembly of ODA type 2. Such a staining pattern was so far not observed in patients with DNAH5 mutations but only in patients carrying DNAH5 mutations (21).

The primary goal of current therapy for patients with PCD is prevention of progressive lung damage (27). The strict treatment of infections of the upper and lower airways, appropriate antibiotics are able to suppress the usage of premature termination codons by permitting translation to continue to the normal termination of translation (28, 29). Clinical studies using pharmacogeno- therapy in patients with cystic fibrosis carrying premature nonsense mutations in the CFTR gene provided compelling evidence that the aminoglycoside antibiotics are able to suppress the usage of premature termination codons by permitting translation to continue to the normal termination of translation (28, 29). Clinical studies using pharmacogeno-therapy to correct the primary defect in these patients with PCD. In future in vitro studies, we will address this question by treating primary human respiratory epithelial cells from patients with PCD carrying nonsense DNAH5 mutations with aminoglycosides. Possibly, this will lead to the development of novel therapeutic strategies in PCD.

A recent work studying a large cohort of patients with PCD for the presence of DNAH mutations shows that mutations in this gene can be identified in 13% of PCD families with ODA defects (M.A. Zariwala and coworkers, submitted for publication). To date, 134 PCD families have been screened for mutations in DNAH5 and recessive mutations were identified in 49% of PCD families with ODA defects (see Figure E4). Thus, although PCD represents a genetically heterogeneous disorder, DNAH5 dysfunction appears to be a major cause of PCD with ODA defects.

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