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DNAI1 Mutations Explain Only 2% of Primary Ciliary Dykinesia

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Key Words
Primary ciliary dyskinesia · Kartagener syndrome · *DNAI1* mutation · Primary ciliary dyskinesia heterogeneity · Intermediate dynein chain

Abstract
Background: Primary ciliary dyskinesia (PCD) is a rare recessive hereditary disorder characterized by dysmotility to immotility of ciliated and flagellated structures. Its main symptoms are respiratory, caused by defective ciliary beating in the epithelium of the upper airways (nose, bronchi and paranasal sinuses). Impairing the drainage of inhaled microorganisms and particles leads to recurrent infections and pulmonary complications. To date, 5 genes encoding 3 dynein protein arm subunits *(DNAI1*, *DNAH5* and *DNAH11)*, the kinase *TXNDC3* and the X-linked *RPGR* have been found to be mutated in PCD. ***Objectives:*** We proposed to determine the impact of the *DNAI1* gene on a cohort of unrelated PCD patients (n = 104) recruited without any phenotypic preselection. ***Methods:*** We used denaturing high-performance liquid chromatography and sequencing to screen for mutations in the coding and splicing site sequences of the gene *DNAI1*. ***Results:*** Three mutations were identified: a novel missense variant (p.Glu174Lys) was found in 1 patient and 2 previously reported variants were identified (p.Trp568Ser in 1 patient and IVS1+2_3insT in 3 patients). Overall, mutations on both alleles of gene *DNAI1* were identified in only 2% of our clinically heterogeneous cohort of patients. ***Conclusion:*** We conclude that *DNAI1* gene mutation is not a common cause of PCD, and that major or several additional disease gene(s) still remain to be identified before a sensitive molecular diagnostic test can be developed for PCD.

Introduction

Primary ciliary dyskinesia (PCD; OMIM 242650), or immotile cilia syndrome, is a clinically and genetically heterogeneous disease of the ciliated and flagellated structures which mainly follows an autosomal recessive inheritance pattern. The prevalence of PCD is estimated to be 1 in 20,000 live births (1/12,500 to 1/30,000) [1].

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Ciliary function defects in the respiratory system result in chronic sinusitis and upper respiratory tract infections, often leading to nasal polyps and almost always to bronchiectasis [2]. The triad of situs inversus, chronic sinusitis or bronchitis and bronchiectasis, which occurs in 50% of patients, is referred to as Kartagener syndrome (KS; OMIM 244400) [3].

Electron microscopy of cilia from PCD and KS patients detects structural axonemal alterations in more than 60% of patients [4]. The most common ultrastructural abnormalities involve the outer (ODA) or inner (IDA) dynein arms, which are shortened or absent. The dynein arms are multisubunit protein complexes composed of several light, intermediate and heavy chains, which are encoded by distinct loci dispersed throughout the genome. Absent or defective dynein arms are likely to result from a failure in correct assembly of the whole structure, secondary to a mutation in a dynein or other controlling gene(s).

Previous studies have identified the molecular basis of PCD/KS in up to approximately 38% of patients who had mutations in autosomal genes coding for dyneins *(DNAI1, DNAH5, DNAH11)* [5–12]. Another candidate gene, *TXNDC3*, encoding thioredoxin-nucleoside diphosphate kinase, was also recently described [13]. In addition, the gene *RPGR* was proposed to be responsible for an X-linked form associated with retinitis pigmentosa [14]. The several chromosomal loci pinpointed by linkage analysis support the idea that the etiology of the condition is very heterogeneous [15–18].

Of the 5 currently known genes associated with PCD, the axonemal intermediate dynein chain 1 *(DNAI1)* gene has been the most studied [5–7, 11, 14], although mutations in the coding or splicing sequence have been found in only 10% of PCD patients. In the current study we assessed the frequency of mutations in gene *DNAI1* in a large cohort of PCD patients without a selection based on the ultrastructure and/or genotype. The rationale was: (1) the DNAI1 protein is localized within the ODA of the ciliary axoneme arm which is often, but not always, absent or shortened in PCD patients [19]; (2) we had previously identified a small number of families showing absent or abnormal ODA combined with linkage to *DNAI1* locus and mutations in the *DNAI1* gene compared to previous reports; (3) the prevalence of *DNAI1* mutations in PCD found in a number of previous studies might be biased, since a large number of patients was recruited through preselection for linkage to *DNAI1* locus or ultrastructural phenotype.

Methods

Patients

Patients and families (unaffected parents and affected/unaffected siblings; n = 108) were recruited through contacts with pulmonary and ear/nose/throat physicians, and originated from various geographical locations, including Switzerland (n = 50), Italy (n = 32), USA (n = 6), Belgium (n = 5), Spain (n = 3), Canada (n = 2), France (n = 2), Ukraine (n = 2), Brazil (n = 1), Finland (n = 1), Pakistan (n = 1), Syria (n = 1), Sweden (n = 1) and the UK (n = 1). At least 1 of the 3 following criteria were required for inclusion in the cohort of PCD/KS patients: (1) a discrete defect in any of the ciliary ultrastructures (IDA and ODA, nexin links, radial spokes, microtubule configuration) by electronic microscopy, or recurrent upper respiratory tract infections associated with either (2) absence of ciliary movement or (3) situs inversus. Other characteristics were also recorded, such as recurrent pneumonia, bronchiectasis, low nasal nitric oxide measurement, reduced mucociliary clearance and male infertility. Cystic fibrosis was excluded in 32% (n = 35) of the patients by sweat test. If data on ciliary structure and/or function were not available, the presence of recurrent upper respiratory tract infections without situs inversus was not sufficient for inclusion.

One hundred and four cases, mainly composed of Caucasians (91%), met at least 1 of the 3 inclusion criteria and constituted the study population (online suppl. table E1, www.karger.com/doi/10.1159/000128567). Fifty-nine percent had a situs inversus (KS) and ultrastructural information was available for 80% (n = 83) of the patients. Among the patients showing dynein arm defects, 46% had ODA and IDA, 14% ODA alone and 17% had isolated IDA defect. The remaining 19% had other ultrastructural features (described above), while 4% had normal ciliary axoneme ultrastructure.

Among the patients without available ultrastructure analysis (n = 21), all had recurrent upper respiratory tract infections, 14 (46%) were associated with a situs inversus, 4 (19%) had immotile cilia and 2 (10%) had immotile cilia and a situs inversus. Four cases were excluded: all had recurrent respiratory tract infections, but situs inversus was absent (n = 1) or not reported (n = 3) and data on ciliary structure or function were not available (n = 4).

The Swiss Registries for Interstitial and Orphan Lung Diseases (SIOLD) were involved in a nationwide case recruitment within a network of 200 corresponding pulmonary physicians. Written informed consent was obtained from all patients, and the research protocol was approved by the Ethics Committee of the University Hospitals of Geneva.

Mutation Analysis

PCR Amplification

Genomic DNA was isolated from peripheral blood using standard extraction procedures. A specific primer pair was designed for each exon of *DNAI1*, at least 50 bp away from exon limits, to allow amplification and sequence analysis of the entire exon, the flanking intronic splicing sites and adjacent UTR regions (online suppl. table E3, www.karger.com/doi/10.1159/000128567). All amplicons were amplified using a PCR touchdown program (that is, on the first 10 cycles, annealing was decreased at 1°C/cycle starting at 60°C, the 24 remaining cycles were performed at 50°C).

Denaturing High-Performance Liquid Chromatography

DNA heteroduplexes were produced by denaturation of PCR products at 95°C for 10 min, followed by a slow and gradual reannealing of single strands from 95 to 25°C over a 30-min period. Heteroduplexes were distinguished from homoduplexes by denaturing high-performance liquid chromatography (DHPLC) analysis using a WAVE 3500 HT DNA fragment analysis system (Transgenomic, Omaha, Nebr., USA). For each amplicon, a range of partial denaturation temperatures was modeled using the WAVEMaker software (Transgenomic). One to four partial renaturing temperature steps were needed to resolve variants. Five microliters of heteroduplexed PCR fragments was injected into the DNASep cartridge for analysis. DHPLC conditions were first controlled with DNA from normal and affected individuals to assess the quality of the peaks.

Sequence Analysis

All nucleotide changes pointed out by DHPLC variants were resolved by direct sequencing of PCR products. Specifically, all exons were directly sequenced in a total of 41 patients: patients from consanguineous families (n = 7), patients with only 1 mutation found (n = 2), Italian cohort of patients (n = 26) and patients with homozygous regions (n = 6) detected by single nucleotide polymorphism (SNP) genotyping. Whenever a missense variant was found, the possibility that we were dealing with a nonpathological polymorphism was considered by interrogating the SNP database (www.ncbi.nlm.nih.gov/dbSNP/) and verifying the amino acid conservation among other species using the UCSC genome browser (www.genome.cse.ucsc.edu/).

Restriction Analysis

The specific screening for mutation c.48+2_48+3insT (IVS1+2_3insT) was performed as described elsewhere [6] by restriction by *HpaI* of the PCR product amplified with primers X1F and X1R according to the manufacturer's protocol. The fragments of 371 bp (wild-type allele), 179 and 193 bp (mutant alleles) were then resolved in a 2% agarose gel.

SNP Analysis

SNP analysis was performed by minisequencing (Pyrosequencing, Uppsala, Sweden). Genotyping for the polymorphisms rs4879792, rs10738928, rs3793472, rs4878575, rs9657620, rs17353380, rs11999046, rs12002889 and rs12001113 was performed on 20-μl volume PCR amplicons from 50-ng template DNA.

Results

Prior to the current study on our entire cohort of unselected individuals with PCD, a previous mutation screening of gene *DNAI1* had been performed by single-strand conformation analysis (SSCA) on 3 (of 15) families selected, first by the phenotype (absence or abnormal length of ODA) and second by linkage to *DNAI1* gene locus using the microsatellite marker D9S1817 (data not shown). Compound heterozygous *DNAI1* mutations were found (c.[48+2_48+3insT]+[1703C>G]) in 1 family (GVA15; patient No. 6181), while no pathogenic variants were found in the 2 other families.

DNAI1 was investigated for sequence variants in the 20 coding exons and in a minimum of 50 bp, of 3′- and 5′-untranslated regions as well as intronic sequences adjacent to acceptor and donor splicing sites. The strategy used to characterize *DNAI1* gene mutation on our entire cohort of PCD/KS patients and the respective results are shown in figure 1.

Overall, we identified 5 variants (table 1). The nonsynonymous p.Val335Ile in exon 11 (41 patients; 82/208 alleles) and p.Ala8Ser in exon 1 (2 patients; 4/208 alleles) were believed to be SNPs rather than pathological variants, as reported previously [5, 6]. Among the 3 other variants, the well-known recurrent mutation c.48+2_48+3insT (IVS1+2_3insT), which affects donor splicing of intron 1, was found in 4 alleles carried by 3 unrelated patients. This most prevalent mutation in *DNAI1* was detected in the heterozygous state in patients No. 6181 (family GVA15, as seen in previous analysis, data not shown) and No. 6177 (family GVA14). Patient No. 8227 was homozygous for the c.48+2_48+3insT mutation.

In exon 17, we have identified the previously reported c.1703G>C transversion that would result in the missense change p.Trp568Ser at protein level [5, 7]. The substitution of this amino acid that is highly conserved throughout evolution is predicted to alter normal folding of the protein, leading to loss of function [7]. The mutation status of patient No. 6181 [family GVA15, compound heterozygous c.[1703C>G]+[48+2_48+3insT] previously identified by SSCA on preselected families (data not shown), was also detected with DHPLC screening.

A novel variation was identified in exon 7 (patient No. 6127, family GVA02): the transition c.520G>A results in a missense mutation at codon 174 (p.Glu174Lys; fig. 2a). Glutamic acid at position 174 appears to be highly conserved across mammalian species (www.genome.cse.ucsc.edu/). It is localized 4 amino acids upstream of the WD40-like domain (fig. 2b). The underlying common function of all WD-repeat proteins is to coordinate multiprotein complex assembly, as it happens in dynein arms. To verify whether this variant could be a nonpathogenic polymorphism, we screened a control population of mainly Caucasian origin. No carrier individual (180 control chromosomes tested) was found, further supporting the hypothesis that the p.Glu174Lys missense is a disease-causing mutation.

In order to verify that no homozygous mutations had been missed, since standard DHPLC conditions do not

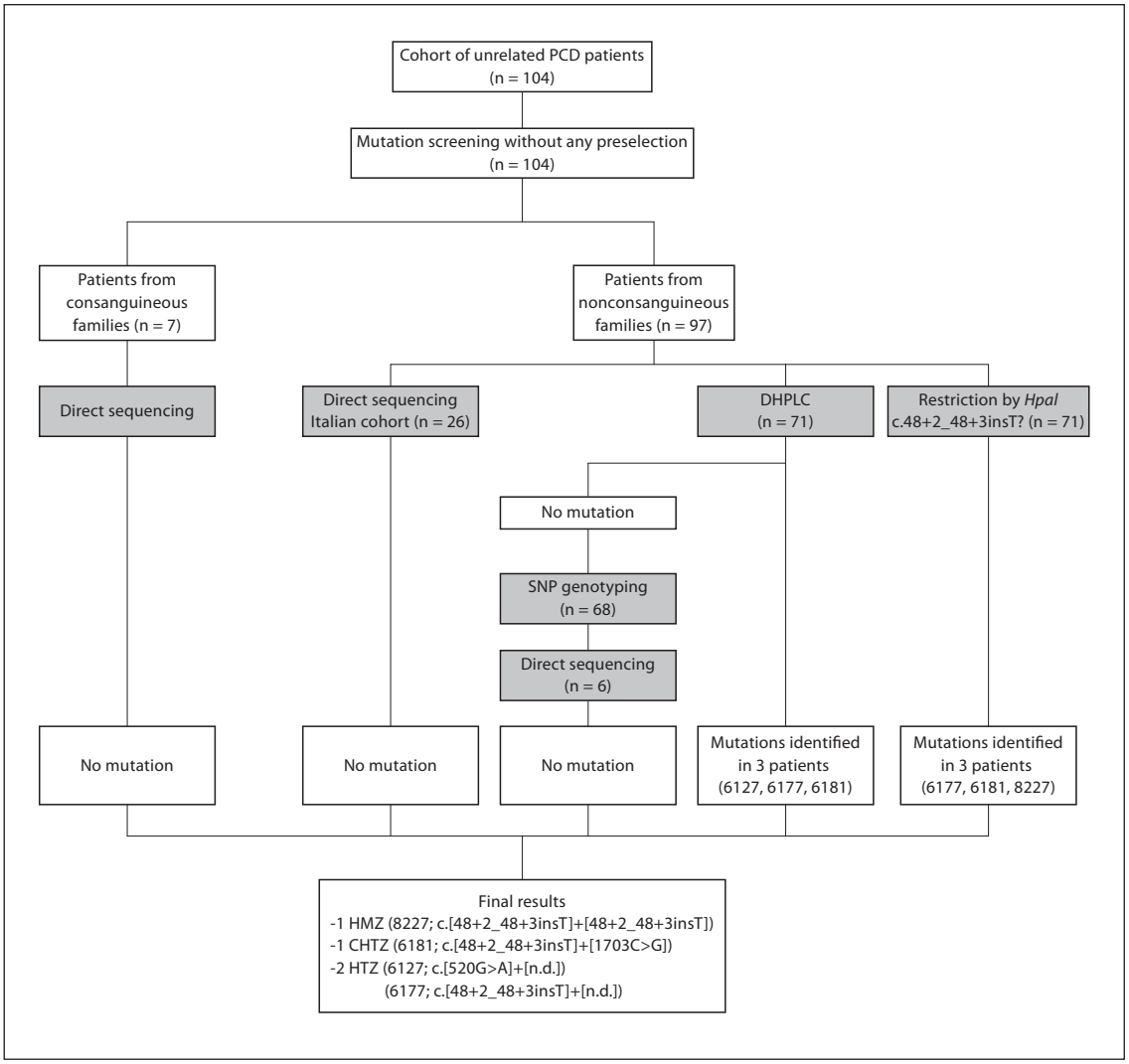

Fig. 1. Flow chart of the strategy used to study *DNAI1* mutation on PCD/KS patients. HMZ = Homozygote; HTZ = heterozygote; CHTZ = compound heterozygote; n.d. = not determined.

Table 1. Mutations of gene *DNAI1* found in patients with PCD

Family No.	Patient No.	Location	DNA change	Protein change	Ciliary utrastructural defect
GVA02	6127	[exon 7]+[?]	c.[520G>A]+[?]	p.[Glu174Lys]+[?]	ODA+IDA
GVA14	6177	[intron 1]+[?]	c.[48+2_48+3insT]+[?]	p.[Ser17fsX25]+[?]	ODA
GVA15	6181	[intron 1]+[exon 17]	c.[48+2_48+3insT]+[1703C>G]	p.[Ser17fsX25]+[Trp568Ser]	ODA
GVA70	8227	[intron 1]+[intron 1]	c.[48+2_48+3insT]+[48+2_48+3insT]	p.[Ser17fsX25]+[Ser17fsX25]	ODA

All patients were Caucasians. Mutation coding is described according to latest recommendations of the Human Genome Variation Society (http://www.hgvs.org/mutnomen/).

detect homozygous deletion, we firstly checked for the occurrence of c.48+2_48+3insT in our entire cohort by an independent restriction analysis [6]. We confirmed the mutation status of the 2 heterozygous patients previously identified by DHPLC (No. 6177 and No. 6181) as well as of patient No. 8227 homozygous for the c.48+2_48+3insT. No additional patients carry this mutation, confirming the previous analysis findings. Next, we genotyped SNPs of the region encompassing *DNAI1* gene in DNA of the individuals (n = 68) in which no heterozygous variants had been identified and of individuals who had not been fully analyzed by direct sequencing (Italian cohort and issued from consanguineous marriages). According to the pattern of conserved haplotypes in the Caucasian population, as demonstrated in the HapMap study on CEU sample (www.hapmap.org) [20], the whole length of *DNAI1* is in linkage disequilibrium. Nine tag SNPs and 2 informative SNPs representing the whole linkage disequilibrium block (online suppl. figure E2, www.karger.com/doi/10.1159/000128567) were selected to determine which individuals were suspected to be homozygous (online suppl. table E4, www.karger.com/doi/10.1159/000128567). Overall, with SNP results and other heterozygous variants detected in the mutation analysis course, 98 patients were found to be heterozygous in at least 1 variant located within the *DNAI1* gene. Furthermore, all remaining individuals (n = 6) not showing heterozygosity in this region were directly sequenced for the 20 exons of *DNAI1*. No additional mutation was detected.

In summary, we have identified 6 altered alleles in the *DNAI1* gene of 4 unrelated patients within our cohort of 104 individuals (208 alleles) with PCD. Of these, 1 patient was a homozygous carrier of the c.48+2_48+3insT mutation, 1 was a compound heterozygous carrying both the c.48+ 2_48+3insT (p.Ser17fsX25) and c.1703G>C (p.Trp568Ser) and 2 patients had monoallelic mutations (either c.48+2_48+3insT or c.520G>A). In order to find the second mutant allele, *DNAI1* exons and at least 50 bp upstream and downstream of respective splicing sites were directly sequenced. No additional mutation was identified.

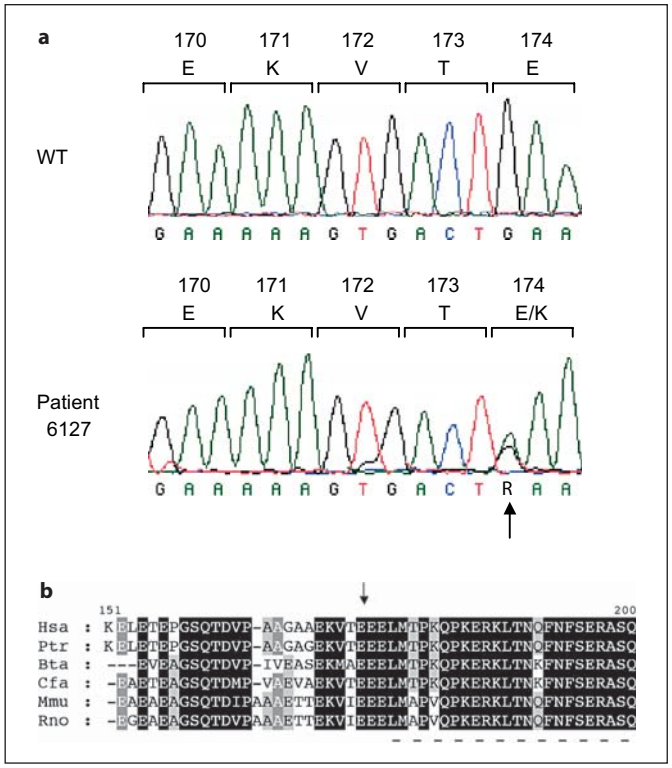

Fig. 2. Sequence analysis of novel mutation in exon 7 of *DNAI1*. **a** Partial chromatogram of DNA sequencing of exon 7 showing the change at nucleotide c.G520 (arrow) denoting heterozygous missense mutation (p.Glu174Lys) in patient No. 6127 (lower panel) by comparison with wild type (upper panel). WT = Wild type. **b** Partial alignment of the DNAI1 amino acid sequence (residues 151–200) showing the phylogenic conservation of the region where the missense mutation p.Glu174Lys occurs (arrow). Highly conserved residues between the different species are denoted by a blackened background, whereas similar residues are shaded. The dotted line denotes the beginning of the WD40-like region. Hsa = *Homo sapiens;* Ptr = *Pan troglodytes;* Bta = *Bos taurus;* Cfa = *Canis familiaris;* Mmu = *Mus musculus;* Rno = *Rattus norvegicus.*

Discussion

The main finding of the present study is the determination of the frequency of *DNAI1* mutations associated with PCD in our 104 unrelated patients which were also unselected, on the basis of phenotype, genotype or geographical origin. We found that 2% of our cohort of PCD patients has disease-causing mutations in *DNAI1,* while the frequency of patients with at least 1 mutated allele was 4%. This contrasts with previous data in which 10% of patients were found to carry mutations in *DNAI1* [11].

In 2 of these 4 patients, we could not find the second mutant allele, although all *DNAI1* exons were subsequently investigated by direct sequencing. Such monoallelic mutation has been previously described [7, 11]. An alternative could be that the second mutation might be located in noncoding regions of gene *DNAI1* (5′-UTR, 3′-UTR, promoter or intron), which have not been fully screened, as well as extended deletion not detectable by

techniques used in this study. The second possibility is that *DNAI1* is not the gene causing PCD in these 2 patients. A third hypothesis is a possible digenic inheritance or triallelism as described in a few families with Bardet-Biedl syndrome [21]. Although no evidence for such digenic or multiallelism inheritance has been described yet in PCD, this hypothesis cannot be completely ruled out for these 2 patients (No. 6127 and No. 6177).

The majority of patients (80 of 104) had abnormal ultrastructure defects of the cilia and the cohort is thought to be representative of PCD population, since the rate of occurrence of specific ultrastructural defect did not deviate from previously reported studies.

Eight percent of patients (4 of 50) with either ODA or combined ODA and IDA defect had *DNAI1* mutation(s). We did not find any *DNAI1* mutation in patients without ODA defect, which is in agreement with previous reports [6, 11].

Overall, the frequency of *DNAI1* mutation among our cohort is 4% (4/104 patients and 6/208 alleles) for patients carrying at least 1 mutated allele and 2% for patients with 2 mutated alleles. This contrasts with the study of Zariwala et al. [11] (179 unrelated individuals), in which 10% of patients were found to carry mutations in *DNAI1*. Since their as well as our cohorts were mainly Caucasian, a founder effect and cohorts with different balances of geographic origin could explain the deviation in the frequency of *DNAI1* mutations observed.

Thus, the design of a clinical molecular panel for diagnosis, if it includes the entire *DNAI1* gene sequence, should also rely on a set of additional genes, a list of which is to date far from being complete. For *DNAI1,* a screening for the most prevalent known mutations up to date, that is, c.48+2_48+3insT and mutation in exons 13, 16 and 17, would be enough, since these mutations account for more than 80% of all patients with a *DNAI1* alteration. *DNAH5* should be included as well, since a recent report described that 28% of PCD patients with ODA defect carry a mutation in this gene within a cluster of 5 exons [11].

Several genes for axonemal dynein genes *(DNAH7, DNAH17, DNAH9, DNAL1, DNAH3, DNAI2, LC8, HP28, DNAL4)* as well as other reasonable candidates for PCD (with or without situs inversus) [8, 22–25] have been screened for mutations in the past years, but none of these were confirmed as being a significant cause of PCD. In some of these candidate genes, potential pathogenic variants were identified as in *DNAH9* [26], but their role could not be confirmed, since either they might represent rare polymorphisms or the second putative mutant allele was not found. Since the 5 currently known PCD genes have been investigated with low success rate, the etiology remains unknown in a large proportion of the studied cohorts (60–90%), and there is still a strong need to investigate new candidate genes for PCD/KS.

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