DNAI2 mutations can cause primary ciliary dyskinesia with outer dynein arm defects

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Abstract

Primary ciliary dyskinesia (PCD) is a genetically heterogeneous disorder characterized by recurrent infections of the airways and randomization of left/right body asymmetry. The phenotype results from dysfunction of motile cilia of the respiratory epithelium and the embryonic node. Dysmotile sperm tails often cause infertility in male PCD patients. Underlying ultrastructural defects frequently involve outer dynein arms (ODA), which are responsible for generation of cilia movement. We recently showed that recessive mutations of DNAH5 encoding a heavy dynein chain are frequently found in PCD with ODA defects. Genes (DNAI1, TXNDC3, DNAH11) encoding for other ODA components can also account for PCD. Here, we analyzed the protein expression of the ODA intermediate chain DNAI2 and found sub-localization throughout respiratory cilia and sperm tails. Mutational screening of 105 PCD families revealed in one affected a homozygous mutation within the facultative splice acceptor site of exon 4. RNA studies confirmed absence of exon 4 in all transcripts predicting a premature stop codon. Consistently mutant respiratory cells lacked DNAI2 expression and exhibited ODA defects by electron microscopy. High-resolution immunofluorescence imaging demonstrated mis-localization of DNAH5 indicating that DNAI2 is essential for assembly of these ODA components. In addition we report homozygous loss-of-function DNAI2 mutations located within the obligatory splice donor site of exon 11 in four affected individuals with ODA defects originating from a consanguineous PCD family that showed significant linkage to the DNAI2 locus in a total genome scan. In summary, we provide the first evidence that DNAI2 can account for PCD with ODA defects.
Introduction

Primary ciliary dyskinesia (PCD, MIM 242650), also known as immotile cilia syndrome (ICS) is a rare, usually autosomal recessive, genetic disorder affecting ciliary movement, with an incidence of 1 in 20,000-30,000 (1). Motile cilia covering the epithelia of the upper and lower respiratory tract constantly move inhaled particles, cell debris and microbes towards the throat (2). Due to the lack of mucociliary clearance PCD patients suffer from recurrent infections of the upper and lower respiratory tract that can cause permanent lung damage such as bronchiectasis (3,4). Dysfunction of nodal cilia during early embryogenesis causes randomization of left/right body asymmetry (5), which explains why approximately half of affected PCD individuals have situs inversus (6). The association of PCD and situs inversus is also referred to as Kartagener syndrome (KS, MIM 244400). Male PCD patients often have reduced fertility, which is due to dysmotile sperm tails that have an ultrastructure resembling that of respiratory cilia.

Cilia and flagella are hair-like structures extending from the cell surface (7). Most motile cilia, such as respiratory cilia, consist of nine peripheral doublets surrounding two central tubules (9+2 axoneme). The 9+2 configuration has been preserved throughout evolution, but there are also motile cilia with a 9+0 configuration e.g. nodal cilia. A number of multiprotein complexes like radial spokes, nexin links, central sheath and dynein arms interconnect the different components. Outer dynein arms (ODAs) are connected to the peripheral microtubule A and generate motion by ATP-dependent reactions. The outer dynein arms are complex macromolecular assemblies containing different polypeptide chains classified according their sizes in heavy (400-500 kDa), intermediate (45-110 kDa) and light
chains (8-55 kDa). The dynein heavy chains form the globular heads and the stem of the complexes and contain the ATPase and microtubule motor domains.

We recently showed that recessive mutations of DNAH5 encoding a heavy dynein chain are found in approximately 50% of PCD individuals with ODA defects (8,9). DNAI1 mutations were reported in 13% of PCD patients with defined ODA defects (10,11). Other genes (TXNDC3, DNAH11) (12,13,14) encoding also for ODA components only rarely account for PCD. Here, we studied DNAI2, the human ortholog of Chlamydomonas intermediate ODA chain IC69 comprising 14 exons extending over 39 kb genomic distance (15).

We analyzed the expression of the DNAI2 chain in respiratory cells and sperm cells, and evaluated the DNAI2 gene as a possible candidate gene for PCD. After identification of recessive loss-of-function DNAI2 mutations in two PCD families we analyzed the effect of DNAI2 deficiency on the assembly of ODA complexes using transmission electron microscopy and high-resolution immunofluorescence imaging in respiratory cells of this patient.
Results

Sub-cellular localization of DNAI2 in respiratory cells and sperm cells

Based on homology to the *Chlamydomonas* intermediate ODA chain IC69 we assumed similar functions for DNAI2. Consistent with a possible function in cilia or flagella motility, *DNAI2* expression in respiratory cells and testis was reported previously (15). To further corroborate that DNAI2 indeed functions in ODA complexes within respiratory cilia and sperm tails we performed protein expression analyses using monoclonal antibodies directed against DNAI2. To confirm antibody specificity we performed Western blot analyses of extracts from human respiratory cell cultures. Anti-DNAI2 antibodies detected a single band of ~69 kD which corresponds to the predicted molecular weight of DNAI2 (Figure 1A). We next analyzed the sub-cellular localization of DNAI2 in human respiratory and sperm cells using high-resolution immunofluorescence imaging. As controls we used antibodies against the dynein axonemal heavy chain DNAH5. DNAI2 staining was observed throughout all analyzed respiratory ciliary axonemes and sperm flagella (Figure 1B and C) indicating that assembled ODA complexes along the entire length of these axonemes probably contain DNAI2. Based on this finding we considered *DNAI2* a strong candidate gene for PCD.

Mutational analysis of *DNAI2*

Sequencing of all 14 coding exons including the exon/intron boundaries in 105 affected (including 7 Hungarian families) originating from unrelated PCD families revealed a novel *DNAI2* variant in a single Hungarian PCD patient (Fig. 2A). A facultative splicing mutation IVS3-3T>G located within the evolutionarily conserved
(~80% conservation) splice acceptor site of exon 4 was absent in 236 Caucasian control chromosomes. The mutation co-segregated with the disease status in the Caucasian family OP42 and was present in the affected in the homozygous state consistent with homozygosity by descent (Fig. 2B). Previous sequencing of all coding DNAH5 exons in OP42 was normal (9). Interestingly, clinical history did not reveal any known consanguinity of the parents. However, because both parents originate from the same geographical region a distant relationship of both parents is possible. The clinical findings of the patient comprised chronic otitis media, recurrent bronchitis and pneumonia, bronchiectasis and situs inversus consistent with Kartagener syndrome. To test whether the IVS3-3T>G mutation is especially prevalent in East Europe we analyzed 125 Polish PCD patients for presence of this sequence variant. However, mutational analysis was normal.

A total genome scan identified linkage to chromosome 17 in a consanguineous Iranian Jewish kindred. A 21.4 cM (9.6Mb) region of homozygosity shared between the four affected offspring was identified on chromosome 17q25.1 across the DNAI2 gene between flanking markers rs755424 and rs938350, with a maximum lod score of 3.97 (Fig. 3A) at marker rs1872076 which is located 780 kb centromeric to the start of the DNAI2. Sequencing of all 14 DNAI2 exons found a mutation, IVS11+1G>A, affecting the obligatory (100% sequence conservation) donor splice site of exon 11. Genotype analyses for this loss-of function mutation that predicts a premature stop codon (M499IfsX50) showed co-segregation with the disease status in both nuclear families of the extended kindred (Fig. 3C). All analyzed parents (149M and F, 150M and F, Fig. 3B) and one of the unaffected offspring (149Na, Fig. 3B) are heterozygous carriers (Fig. 3C). The unaffected offsprings 149Nb and 150Nb (Fig. 3B) are homozygous carriers for the wildtype allele (Fig. 3C).
Consistent with homozygosity by descent in the affected patients 149Pa, 149Pb, 150Pa and 150Pb the mutation was present in a homozygous state (Fig. 3C). The clinical findings of the affected patients include neonatal pneumonia, recurrent rhinitis and sinusitis, recurrent otitis media and hearing deficits, chronic cough, bronchiectasis. Two affected have normal situs solitus (149Pb, 150Pb) and two exhibit situs inversus (149Pa, 150Pa) consistent with Kartagener syndrome and randomization of left/right body asymmetry. In one of the affected males infertility was observed.

The IVS3-3T>G mutation results in abnormal splicing

We considered the IVS3-3T>G variant to represent a mutation, because this homozygous DNA exchange was absent in the Caucasian control population and co-segregated with the disease status. To confirm that the DNA variant located within the splice acceptor site of intron 3 indeed results in aberrant splicing we performed RT-PCR. Cloning and subsequent sequencing of the PCR products confirmed in all transcripts of the affected individual OP42-II2 the absence of exon 4 and out of frame fusion of exon 3 to exon 5 (Fig. 2C). RT-PCR analysis in control RNA elicited wild-type transcripts which had exon 4 present. We conclude that the mutant splice site is not recognized by the splicing machinery resulting in skipping of exon 4 and this predicts early premature termination of translation because of out-of-frame transcripts (I116GfsX54).

Molecular defects caused by the DNAI2 mutation

Next we analyzed DNAI2 expression in respiratory cells that were obtained by nasal brushing biopsy from patient OP42-II2 harbouring the novel splicing mutation.
Consistent with a loss-of-function mutation, all analyzed cells lacked DNAI2 expression (Fig. 4) suggesting this is a null allele and confirming the pathogenic significance of the detected mutation. As a control we stained cilia with an antibody directed against the inner dynein arm light chain Dnali1, which is not altered by an ODA defect. We have previously shown that respiratory cilia contain at least two distinct ODA types: type 1: DNAH9-negative and DNAH5-positive (proximal ciliary axoneme); type 2: DNAH9- and DNAH5-positive (distal ciliary axoneme) (16). High-resolution immunofluorescence imaging to detect the ODA heavy dynein chain DNAH5 in respiratory cells from patient OP42-II2 showed an aberrant expression pattern. DNAH5 proteins were undetectable in the ciliary axonemes of these PCD patients (Figure 5B). Thus, we conclude that absence of DNAI2 prevents correct assembly of both ODA complexes (type 1 and type 2) in respiratory cells from patient OP42-II2. Consistent with this finding transmission electron microscopy of respiratory cilia from patient OP42-II2 detected in all observed cross-sections ODA defects on the ultrastructural level (Fig 5D). In transmission electron microscopy cross-sections of respiratory cilia of the patient 150Pb carrying the IVS11+1G>A mutation ultrastructural ODA defects were also present (Fig. 5E).

**Aberrant DNAI2 localization in respiratory cells harboring mutations in DNAH5 and DNAI1 encoding ODA components**

Following the demonstration that DNAI2 deficiency can result in complete absence of DNAH5 from respiratory ciliary axonemes, we investigated the effect of DNAH5 mutations on DNAI2 expression. For this purpose we used respiratory cells from PCD patients carrying DNAH5 mutations that we reported previously (16). In respiratory cells from patient F661 carrying compound heterozygous DNAH5
mutations 4361G>A (exon 28) and 8910+8911delAT>insG (exon 53), we found complete absence of DNAI2 from all analyzed respiratory ciliary axonemes (Figure 6B). This indicated that just as DNAI2 is essential for correct assembly of DNAH5, DNAH5 is essential for correct assembly of DNAI2, in both types of ODA complexes.

Previously we demonstrated that in respiratory cells of patients carrying the compound heterozygous DNAI1 mutations 219+3insT / W568X, assembly of proximal ODA complexes (type 2) in the proximal ciliary axoneme is at least partially preserved, because we found some residual localization of DNAH5 in this proximal ciliary compartment, whereas DNAH5 was absent from the distal ciliary axoneme (16). To investigate whether DNAI2 shows a similar localization pattern as DNAH5 in DNAI1 mutant respiratory cells, we analyzed respiratory epithelial cells from patient OP121-II1 carrying the homozygous 219+3insT DNAI1 mutation. Consistent with our previous finding that in DNAI1 mutant cells assembly of proximal (type 2) ODA complexes is at least partially preserved, we found in all analyzed respiratory cells moderate DNAI2 staining in the proximal part, but absent staining in the distal part of the ciliary compartment (Fig. 6C).
Discussion

Outer dynein arm complexes are large multimeric protein complexes comprising light, intermediate and heavy dynein chains, which are responsible for the generation of the cilia beat (17). Recessive mutations of genes encoding the outer dynein arm components Dnah5, DnaI1, Txndc3 and Dnah11 can cause primary ciliary dyskinesia (8-14). Based on this observation we considered the outer dynein arm intermediate chain gene DNAI2 a functional candidate for primary ciliary dyskinesia (15). Direct sequencing of PCR amplified DNAI2 exons and adjacent intronic sequences in 105 patients identified in one Hungarian patient homozygous DNAI2 mutations affecting the splice acceptor site (Fig. 2A). RNA analysis demonstrated that this splicing mutation indeed results in skipping of exon 4 and an out-of-frame transcript (Fig. 2C) with an early premature stop codon (I116GfsX54). Thus, the mutation probably results in a transcript lacking more than two thirds of the C-terminal protein, which also comprise the functional WD protein domains important for interaction with other proteins. Consistent with the findings obtained by RNA studies we demonstrate lack of DNAI2 protein expression using anti-DNAI2 antibodies (Fig. 4). This suggests that the mutation creates a null allele, possibly due to nonsense mediated decay of the severely truncated protein (18). Furthermore, we found in four patients from a consanguineous Iranian Jewish kindred homozygous loss-of function mutations (IVS11+1G>T) affecting the donor splice site of exon 11 (Fig. 3). DNAI2 mutations appear to result in randomization of left/right body asymmetry, because of the five affected individuals we describe carrying DNAI2 mutations, two exhibited situs solitus and three exhibited situs inversus. Thus, we provide the first evidence that recessive DNAI2 mutations can cause PCD. Considering the high number of PCD individuals screened for the presence of DNAI2
mutations, we consider this gene to be a rare cause of PCD. However, we cannot rule out presence of DNAI2 mutations in non-coding regions of the gene. Other rare causes for PCD are recessive mutations in DNAH11 and TXNDC3. In each of these genes so far mutations were only reported in a few individuals.

Northern blot analysis showed that the DNAI2 gene is strongly expressed in trachea and testis, which is consistent with the observed disease phenotype in primary ciliary dyskinesia (15,19). However, protein expression of DNAI2 has not so far been analyzed. Here, we report DNAI2 protein expression in respiratory cilia and sperm flagella consistent with a functional role of DNAI2 within the ciliary axoneme.

We have previously shown that ODA complexes vary in their composition along the respiratory ciliary axoneme and the axoneme of the sperm tail, and that this composition also differs between these two cell types (16). Respiratory cilia contain at least two distinct ODA types: type 1: DNAH9-negative and DNAH5-positive (proximal ciliary axoneme); type 2: DNAH9- and DNAH5-positive (distal ciliary axoneme). To determine the sub-cellular localization of DNAI2 in various cell types carrying motile cilia or flagella we used specific monoclonal mouse antibodies directed against human DNAI2 for high-resolution immunofluorescence microscopy (Fig. 1A). We demonstrated localization of DNAI2 throughout the entire length of axonemes from respiratory as well as sperm tails (Fig. 1B and C). This finding indicates that DNAI2 is probably present in both ODA (type 1 and 2) complexes.

Interestingly, mutations of the distinct genes encoding ODA components do not result in identical defects. We have shown that DNAH5 and DNAI1 mutations regularly result in absent and/or shortened ODAs visualized by transmission electron microscopy (8,9,11,20,21). In contrast the DNAH11 mutations reported so far do not result in ultrastructural defects (13,14), and TXNDC3 mutant cilia exhibit both normal
and abnormal cilia cross-sections (12). To determine the effect of DNAI2 mutations on axonemal ultrastructure we performed transmission electron microscopy. All visualized cross-sections showed ODA defects (Figure 5D and E) resembling defects identified in patients with DNAH5 mutations (8). To analyze this further, we performed high-resolution immunofluorescence analysis in respiratory cilia from the patient OP42-II2 with DNAI2 mutations. Consistent with deficient assembly of both ODA types 1 and 2, we found absence of the ODA heavy chain DNAH5 from the entire ciliary axoneme (Fig. 5B), indicating that DNAI2 is essential for axonemal assembly of the ODA heavy chain DNAH5 in the analyzed respiratory cells of patient OP42-II2. This observation resembles findings reported in respiratory cilia of patients carrying DNAH5 loss-of-function mutations (9,16). Therefore, we also analyzed respiratory cilia from patients with DNAH5 mutations for DNAI2 expression. DNAI2 expression within the analyzed respiratory cilia was completely absent (Fig. 6B), demonstrating in a complementary manner that DNAH5 is also essential for the axonemal assembly of the ODA protein DNAI2 within ODA types 1 and 2. Furthermore, we analyzed respiratory cilia from a patient carrying a homozygous DNAI1 mutation for DNAI2 localization. DNAI2 expression was restricted to the proximal part of the respiratory cilia and was completely missing in the distal part of the ciliary axoneme (Fig. 5C). We previously reported a similar staining pattern for DNAH5 in DNAI1 mutant cells (16). These findings indicate that mutant DNAI1 in these cells predominantly inhibits axonemal assembly of distally localized ODA types, while proximal ODAs can at least become partially assembled.

Previous findings in Chlamydomonas reinhardtii are consistent with an evolutionarily conserved functional role for DNAI2. Fowkes et al. analyzed the preassembly and stability of ODA complexes in the cytoplasm of wildtype and 11
outer dynein arm assembly (oda) mutant strains (22). The *Chlamydomonas oda6/IC69* gene is the ortholog of *DNAI2* and in the *oda6* mutant strain the ODA complexes are disrupted, indicating the importance of *DNAI2/oda6* for ODA assembly. Furthermore, it was found that both *Chlamydomonas* intermediate chains, IC69 and IC78, form a heterodimer required for stability of the ODA complex (22).

In summary, recessive mutations of *DNAI2* can cause PCD. This finding is consistent with the hypothesis of extensive genetic heterogeneity in primary ciliary dyskinesia (23).
Material and Methods

Immunoblotting

Protein extracts were prepared from human respiratory epithelial cell cultures according to previously published procedures (15,24). Proteins were separated on a NuPAGE 4-12% bis-tris gel (Invitrogen, Karlsruhe, Germany) and blotted onto a PVDF membrane (Amersham). The blot was processed for ECL plus (Amersham) detection using DNAI2 (1:1000) and anti-mouse-HRP (1:5000) antibodies (Santa Cruz, Heidelberg, Germany).

Immunofluorescence analysis

Respiratory epithelial cells were obtained by nasal brush biopsy (cytobrush plus, Medscand Malmö, Sweden) and suspended in cell culture medium. Sperm cells were washed with phosphate buffered saline. Samples were spread onto glass slides, air dried and stored at -80°C until use. Cells were treated with 4% paraformaldehyde, 0.2% Triton-X 100 and 1% skim milk prior to incubation with primary (at least 2 hours) and secondary (30 minutes) antibodies at room temperature. Appropriate controls were performed omitting the primary antibodies. Polyclonal rabbit anti-DNAH5 and anti-Dnali1 antibodies were described previously (15,25). Mouse anti-acetylated-α-tubulin antibodies were obtained from Sigma (Taufkirchen, Germany), monoclonal mouse anti-DNAI2 antibodies from Abnova Corporation (Taiwan) and polyclonal rabbit anti-α/β-tubulin from Cell Signaling Technology (USA). Highly cross adsorbed secondary antibodies (Alexa Fluor 488, Alexa Fluor 546) were obtained
from Molecular Probes (Invitrogen). DNA was stained with Hoechst 33342 (Sigma). Confocal images were taken on a Zeiss LSM 510 i-UV.

**Patients and families**

Signed and informed consent was obtained from patients fulfilling diagnostic criteria of PCD (2) and family members using protocols approved by the Institutional Ethics Review Board at the University of Freiburg and collaborating institutions. We studied DNA from a total of 105 PCD patients originating from unrelated families. In addition we analyzed a consanguineous kindred by total genome scan and mutational analysis (Fig. 3).

**Linkage analysis**

A genome-wide linkage scan was carried out at the Wellcome Trust Centre for Human Genetics, Oxford, using the Illumina Linkage IV Panel of 6008 SNPs. In each sample 98-99% of the SNPs were successfully typed. Multipoint linkage analysis was performed using GeneHunter version 2.1r5, assuming autosomal recessive inheritance, a disease allele frequency of 0.007 and complete penetrance. DNA samples from the family 149 and 150 parents and affected (in total n=8) but not from unaffected offspring were typed in this scan.

**Mutational Analysis**

Genomic DNA was isolated by standard methods directly from blood samples or from lymphocyte cultures after Epstein-Barr virus transformation. Amplification of 14 genomic fragments comprising all 14 exons of DNAI2 was performed in a volume of
50 µl containing 30 ng DNA, 50 pmol of each primer, 2 mM dNTPs, and 1.5 U Taq DNA polymerase (Eppendorf, Hamburg, Germany). PCR amplifications were carried out by means of an initial denaturation step at 94°C for 4 min, and 33 cycles as follows: 94°C for 30 sec, 53-64°C for 30 sec, and 72°C for 60 sec., with a final extension at 72°C for 10 min. PCR-products were verified by agarose gel electrophoresis, column purified (Genomed, Loehne, Germany) and sequenced bi-directionally by using BigDye Terminator v3.1 Cycle Sequencing Kit (Perkin Elmer). Samples were separated and analyzed on an Applied Biosystems 3730xl DNA Analyzer. Sequence data were evaluated using the Codoncode software (CodonCode Corporation, Dedham, USA). To aid identification of frequent polymorphisms we sequenced samples from 26 Caucasian healthy controls. In the family with the affected carrying the novel DNAI2 mutation also other family members were analyzed. We screened additional 236 control chromosomes originating from healthy Caucasians for the presence of the novel DNAI2 mutation. Segregation analyses and screening of controls were performed by restriction analysis and/or sequencing.

**cDNA Analysis of splicing-mutations**

Total RNA was isolated from lymphocytes from patient OP42-II2 and a control using trizol and subjected to first strand cDNA synthesis. To test whether the mutation within the splice acceptor site of intron 3 results in skipping of exon 4, a cDNA fragment comprising exons 3 and exon 5 was amplified (52°C annealing temperature) with primers spanning the junctions of exons 2/3 (Ex2/3F: 5’-GAA CAC GAG GCC AAC TCA G-3’) and exon 5 (Ex5R: 5’-GCT GAA AAT CCA AGC AGG AG-3’) respectively. Products were subjected to second round amplification under
identical conditions. RT-PCR analysis of the control showed a wild-type allele (351bp) and in patient OP42-II2 a single variant allele (229bp) was detected. PCR products were cloned into pCRII (Invitrogen) and subsequently sequenced.

**Transmission electron microscopy**

The biopsies were taken from the middle turbinate. The sample of nasal mucosa was fixed in 2.5% glutaraldehyde in 0.1M sodium cacodylate buffer at 4°C, washed overnight and postfixed in 1% osmium tetroxide. After dehydration, the samples were embedded in a propylene oxide / epoxy resin mixture. After polymerisation several resin sections were cut using an ultra-microtome. The sections were picked up onto copper grids. The sections were stained with Reynold's lead citrate. Transmission electron microscopy was performed with a Philips CM10.
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Conflict of interest statement

There is no conflict in the interests of any author that has contributed to this manuscript and the work contained within it.
References


variant in combination with a nonsense mutation in a member of the thioredoxin family causes primary ciliary dyskinesia. *Proc. Natl. Acad. Sci. USA*, **104**, 3336-3341


**Figure legends**

**Figure 1** Specific antibodies localize the human axonemal outer dynein arm intermediate chain 2 (DNAI2) to the respiratory ciliary axoneme and sperm tail.  
*(A)* Western Blot analysis (right lane) of protein extracts from human nasal cell culture (left lane, protein standard; middle lane, silver staining). Anti-DNAI2 antibodies specifically detect a single band with the predicted size (~69kD). *(B)* Immuno-fluorescence staining of human respiratory epithelial cells and *(C)* spermatozoa with anti-DNAI2 antibodies (green). DNAI2 is localized throughout all analyzed respiratory ciliary axonemes and sperm flagella. Ciliary axonemes and sperm flagellas were co-stained with antibodies directed against axonemal outer dynein arm heavy chain DNAH5 (red). Note DNAH5 also localizes throughout all respiratory ciliary axonmes, but is only present in the proximal sperm flagella. Overlay and brightfield images are shown on the right side. Nuclei were stained with Hoechst 33342 (blue).

**Figure 2** Recessive loss-of-function *DNAI2* mutations in a patient with Kartagener syndrome.  
*(A)* Sequence chromatograph from the affected individual OP42-II2 showing a homozygous T>G transversion at position -3 of the exon 4 splice acceptor site [IVS3-3T>G]. For comparison the wild-type sequence is shown. *(B)* Segregation analysis of the T>G transversion within the family. Presence of the mutation in the genomic DNA from all four family members was assessed by *MaeI* digestion of PCR products. The affected child (II2) showed a single (536bp) band consistent with the presence of two mutant alleles abolishing the restriction sites in all PCR amplified products. In
contrast in the control subject (wt) all PCR products are completely digested (324bp and 212bp bands). Restriction analyses of the unaffected child (II1), his mother (I2) and father (I1) is consistent with heterozygous carrier status. (C) Results of cDNA analysis to assess the effect of the DNAI2 splicing mutation IVS3-3T>G (346-3T>G). Lower sequence chromatograph depicts the cDNA sequence of a control subject. In the mutant cDNA of the affected individual OP42-II2 (upper sequence) the sequence of exon 4 is absent. Thus, the splicing mutation results in skipping of exon 4 and a frame shift resulting in a premature stop codon (I116GfsX54).

Figure 3 Linkage scan and homozygous loss-of function DNAI2 mutations in a large consanguineous kindred.

(A) Multipoint linkage analysis performed using GeneHunter. A 21.4 cM (9.6Mb) region of homozygosity shared between the affected offspring was identified on chromosome 17q25.1 across the DNAI2 locus (maximum LOD score 3.97). Only the parents and affected children were used in the analysis. Asterix indicates situs inversus. (B) Pedigree of the consanguineous kindred with two related nuclear families. (C) Sequence chromatographs of an affected patient (upper sequence), a heterozygous carrier (middle sequence), and a control individual (lower sequence, wt). The detected loss-of function mutation affects the obligatory splice donor site of exon 11 predicting premature termination of translation (M499IfsX50), and was present in homozygous state in all four affecteds of the kindred. The unaffected off-spring 149Nb and 150Nb are homozygous carriers for the wild type allele. All parents (149M and F, 150M and F) and one of the unaffected off-spring (149Na) are heterozygous carriers of the mutant allele.
**Figure 4** Absence of DNAI2 in respiratory epithelial cells from patient OP42-II2 carrying the *DNAI2* mutation IVS3-3T>G (346-3T>G).

Images of respiratory epithelial cells from a healthy control (A) and from the patient carrying the *DNAI2* mutation (B), co-stained with antibodies directed against outer dynein arm intermediate chain DNAI2 (green) and the inner dynein arm component Dnali1 (red) as control staining. Nuclei were stained with Hoechst 33342 (blue). (A) In control cells DNAI2 localizes along the entire length of the respiratory ciliary axonemes. The yellow co-staining within the ciliary axoneme indicates that both proteins co-localize within respiratory ciliary. (B) In the OP42-II2 patient cells DNAI2 is not detectable consistent with a loss-of-function mutation resulting in failure to produce a functional DNAI2 protein.

**Figure 5** Mis-localization of the outer dynein arm heavy chain DNAH5 in respiratory epithelial cells from patient OP42-II2 with the *DNAI2* mutation IVS3-3T>G (346-3T>G).

Images of respiratory epithelial cells from a healthy control (A) and from the patient carrying the *DNAI2* mutation (B), co-stained with antibodies against acetylated α-tubulin (green) and DNAH5 (red). Nuclei were stained with Hoechst 33342 (blue). (A) In healthy control, DNAH5 localizes along the entire length of the axonemes. The yellow colour within the ciliary axoneme indicates that both proteins co-localize to cilia. (B) In *DNAI2* mutant cells DNAH5 is absent from the ciliary axonemes and accumulates within the apical cytoplasm. (C) and (D) Transmission electron micrographs of cross-sections from respiratory cilia originating from a healthy control (C) and from the DNAI2 patients OP42-II2 (D) and 150Pb (E). In the healthy control,
outer dynein arms are visible ((C), red arrows) while in the patient OP42-II2 (D) and 150Pb (E) outer dynein arms are absent.

**Figure 6** Mis-localization of outer dynein arm chain DNAI2 in respiratory epithelial cells from PCD patients carrying mutations in the outer dynein arm components DNAH5 and DNAI1.

Images of respiratory epithelial cells from healthy control (A) compound heterozygote patient carrying the DNAH5 mutations 4361G>A (exon 28) and 8910+8911delAT>insG (exon 53), (B) and a patient homozygous for the DNAI1 mutation 219+3insT, (C). Cells are co-stained with antibodies directed against DNAI2 (green) and against inner dynein arm component Dnali1 as a control (red). Nuclei were stained with Hoechst 33342 (blue). In DNAH5 mutant cells (B) DNAI2 is absent from the ciliary axonemes indicating that DNAH5 is necessary for DNAI2 assembly. In DNAI1 mutant cells (C) DNAI2 (green) is absent from the distal part of the axonemes but still detectable in the proximal ciliary axoneme. This indicates that mutant DNAI1 inhibits assembly of DNAI2 predominantly in the distal ciliary axonemes.
Figures

Figure 1
Figure 2

A

B

C

intron 3  exon 4

I1  I2  I1  I2  wt

600  500  400  300  200  100

G  T  C  C  C  C  C  C  G  A  G  A  T  C  A  T  G  A

A  I  M  Q  L  G  S  G  P  P  G  N  Q  E

A  I  M  Q  L  G  S  I  M  H  C  I  K  Q

Mael
Figure 3
Figure 4
Figure 5
Figure 6

A  control

B  DNAH5 mutant

C  DNAI1 mutant