

Static Respiratory Cilia Associated with Mutations in *Dnahc11/DNAH11*: A Mouse Model of PCD

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Communicated by Ming Qi

Received 6 May 2011; accepted revised manuscript 16 November 2011.

Published online 18 November 2011 in Wiley Online Library (www.wiley.com/humanmutation). DOI: 10.1002/humu.22001

ABSTRACT: Primary ciliary dyskinesia (PCD) is an inherited disorder causing significant upper and lower respiratory tract morbidity and impaired fertility. Half of PCD patients show abnormal situs. Human disease loci have been identified but a mouse model without additional deleterious defects is elusive. The *inversus viscerum* mouse, mutated at the outer arm dynein heavy chain 11 locus (*Dnahc11*) is a known model of heterotaxy. We demonstrated immotile tracheal cilia with normal ultrastructure and reduced sperm motility in the *Dnahc11^{iv}* mouse. This is accompanied by gross rhinitis, sinusitis, and otitis media, all indicators of human PCD. Strikingly, age-related progression of the disease is evident. The *Dnahc11^{iv}* mouse is robust, lacks secondary defects, and requires no intervention to precipitate the phenotype. Together these findings show the *Dnahc11^{iv}* mouse to be an excellent model of many aspects of human PCD. Mutation of the homologous human locus has previously been associated with hyperkinetic tracheal cilia in PCD. Two PCD patients with normal ciliary ultrastructure, one with immotile and one with hyperkinetic cilia were found to carry *DNAH11* mutations. Three novel *DNAH11* mutations were detected indicating that this gene should be investigated in patients with normal ciliary ultrastructure and static, as well as hyperkinetic cilia.

Hum Mutat 00:1–9, 2011. © 2011 Wiley Periodicals, Inc.

KEY WORDS: mouse and human; primary ciliary dyskinesia (model); *Dnahc11/Dnahc11*

Introduction

Cilia are hair-like membrane bound, microtubule-based structures seen in most cells in the body. Immotile primary cilia and motile cilia both contain a microtubule skeleton or axoneme, comprising nine microtubule doublets equally spaced around the periphery of the cilium. A central microtubule pair is also evident in motile cilia such as those in the respiratory tract. At least 250 proteins are associated with the ciliary axoneme [Dutcher, 1995; Gherman et al., 2006; Ostrowski et al., 2002]. Dynein arms on the peripheral microtubules are mechanochemical ATPases powering ciliary movement [Mitchison and Mitchison, 2010]. Motile cilia have multiple roles in human health and disease including respiratory mucociliary clearance and correct specification of left-right (L-R) patterning during embryonic development [Baker and Beales, 2009].

Static or abnormally motile cilia result in primary ciliary dyskinesia (PCD), a disease estimated to affect between 1 in 15,000–30,000 live births [Barbato et al., 2009]. It is caused by genetically heterogeneous, autosomal recessive mutations and results in defective mucociliary clearance [Bush et al., 2007]. PCD is characterized by chronic upper and lower respiratory tract infections with bronchiectasis becoming more prominent with age [Bush et al., 2007]. Male infertility can result from abnormal sperm motility, the sperm tail being structurally homologous with cilia. Early diagnosis of PCD has been highlighted as being clinically important to reduce both short- and long-term morbidity and allow genetic counseling [Bush et al., 2007]. Ultrastructural defects in the axoneme underlie a significant proportion of PCD cases, most frequently defects of dynein arms [Bush et al., 2007]. However, at least 10% of PCD patients have normal ciliary ultrastructure [Greenstone et al., 1983; Jorissen et al., 2000; Papon et al., 2010] and diagnosis often proves particularly challenging in these patients. Two mutations in the dynein heavy chain gene *DNAH11* (MIM# 603339) were recently described in a family with PCD associated with hyperkinetic cilia but normal ultrastructure [Schwabe et al., 2008]. Four independent cases with different *DNAH11* mutations have also been described [Bartoloni et al., 2002; Berg et al., 2011; Pifferi et al., 2010] although the overall prevalence of *DNAH11* mutations in PCD is unknown.

Motile cilia in the embryonic node drive a leftwards “nodal flow”, directing asymmetric embryonic gene expression and ultimately morphology [Nonaka et al., 2002]. Inverted L-R asymmetry (*situs inversus*) has long been linked to PCD and is found in approximately 50% of patients [Kartagener and Horlacher, 1935]. This

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Contract grant sponsors: Advisory Group for National Specialized Services (AGNSS) (The PCD diagnostic service in Southampton); UK MRC (G0501680, U142670370) (to D.P.N.); the Fondation Boninchi (to L.B.); the Sir Jules Thorn Charitable Trust (to L.B.); the Milena Carvajal Pro-Kartagener Foundation (to L.B.) .

randomization is believed to result from dysfunctional nodal cilia leading to defective nodal flow [Nonaka et al., 2002]. The classical mouse mutant, inversus viscerum (*Dnahc11^{iv}*; *iv*), was originally identified as demonstrating randomized situs [Hummel and Chapman, 1959] and was subsequently shown to be accompanied by immotile nodal cilia and absent nodal flow [Okada et al., 1999]. It results from a missense (E2271K) mutation in the AAA2 domain of *Dnahc11*, the mouse homolog of *DNAH11* [Supp et al., 1997; Supp et al., 1999]. Subsequently, in a morphology-based study, tracheal cilia and sperm were reported to demonstrate normal motility [Handel and Kennedy, 1984]. Other alleles include an engineered null allele *Dnahc11^{tm15sp}* [Supp et al., 1999] and a splicing mutant *Dnahc11^{lrm3}* (*lrm3*) that lacks 50 amino acids from the hinge region [Ermakov et al., 2009] both similarly demonstrate L-R defects, although nodal cilia motility has not been assayed in these alleles.

A number of potential mouse PCD models have been characterized. A significant proportion, including *Dnahc5* (MIM# 603335) [Tan et al., 2007], *Poll* (MIM# 606343) [Kobayashi et al., 2002], and *Dnaic1* (MIM# 604366) [Ostrowski et al., 2010], demonstrate additional defects, particularly hydrocephalus and cardiac anomalies, reducing their viability and raising significant animal welfare issues. The repeated demonstration that mutation of human PCD loci in the mouse results in hydrocephalus has led some to suggest an underlying difference in physiology [Ibanez-Tallon et al., 2002; Ostrowski et al., 2010]. The nm1054 mouse (MGI: Del(1)1Brk), a six-gene deletion, gives rise to PCD-like upper respiratory pathology, lacks cardiac defects, and only shows hydrocephalus on certain genetic backgrounds. These mice, however, show loss of sperm tails and lethal anemia. Transgenic rescue demonstrated *Pcdp1* (MGI: *Gm101*) to underlie the PCD-like symptoms and *Steap3* the lethal anemia [Lee et al., 2008; Ohgami et al., 2005], permitting genetic rescue of the anemia. The *Dnahc1* (MIM# 603332) null results in reduced tracheal cilia and sperm tail motility; however, no analysis of upper respiratory tract pathology was performed making it unclear how well this models PCD. A preliminary analysis of *Ttll1* (MIM# 608955) mutants [Vogel et al., 2010] demonstrates PCD-like upper respiratory pathology, but ciliary motility and morphology has yet to be studied. These data led Ostrowski and colleagues to engineer postnatal deletion of *Dnaic1* (MIM# 604366); deletion at 8–12 weeks of age avoids hydrocephalus. This, however, requires additional experimental intervention and clearly cannot model the condition in juveniles, the stage at which the disease is first evident in humans [Ostrowski et al., 2010]. Whether an easy-to-use mouse model of the respiratory PCD phenotypes exists, lacking additional phenotypes, remains to be determined.

To investigate the potential of *iv* mice as a model of PCD, we examined ciliary function, ciliary ultrastructure, and pathological read-outs of PCD-related disease (including otitis media) that often accompany PCD in humans. Previous studies have suggested normal tracheal ciliary and sperm motility in *iv* mice on the basis of ultrastructure but did not include motility assays [Handel and Kennedy, 1984]. We found tracheal cilia to be static at 37°C, consistent with a PCD phenotype and pathological evidence of PCD-like symptoms in the upper respiratory tract. These data argue that *iv* models aspects of human PCD in the mouse. In parallel to our mouse model analysis, we reviewed the data of consecutive patients referred to our national PCD diagnostic service (2006–2009). Twenty-eight patients were diagnosed with “classical PCD” defined by abnormal ciliary function plus diagnostic ciliary transmission electron microscopy (TEM), and 241 patients had PCD excluded on the basis of light microscopy ± electron microscopy (“not PCD”). Two unrelated patients had a clinical phenotype strongly indicative of PCD

but with normal ciliary ultrastructure. We tested these patients and both were found to carry novel mutations in *DNAH11*.

Methods

Mice

Mice were bred in a specific pathogen free (SPF) environment at MRC Harwell. Inbred *Dnahc11^{iv}* (*iv*) mice are directly descended from the mixed background stock described by Hummel and Chapman [Hummel and Chapman, 1959]. Congenic *iv*/+ mice were derived by 10 generations of backcrosses to C3H/HeH (C3H); C3H-congenic experimental cohorts were from congenic *iv*/+ intercrosses. C3H/HeH congenic *Dnahc11^{lrm3}* (*lrm3*) are as previously described [Ermakov et al., 2009]. SNP genotyping was for the published mutations [Ermakov et al., 2009; Supp et al., 1997]. Samples were collected postmortem, following terminal anaesthesia using sodium pentobarbitone (Euthatal). Control and *iv*/*iv* samples were coded and researchers blinded to both genotype and phenotype (situs). The mouse age, gender, and cohort size is presented in Table 1. Fertility testing was conducted by placing two C57Bl/6J females each with six C3H/HeH congenic *iv*/*iv* males and six +/- sibling control males. Copulation plugs were checked for daily over one week. At 8.5 d.p.c. plugged females were sacrificed, uterine horns dissected, and the number of implanted embryos counted. All males and females used were age matched.

Histopathology

Sections of mouse lung, snout, and middle ear were produced following standard necropsy and histological protocols [Brown et al., 2005; EUMORPHIA Consortium, 2007]. Briefly, trachea and lungs were inflated with 10% neutral buffered formalin (NBF) then immersion fixed along with the head. Decalcification was as described in Parkinson et al. [Parkinson et al., 2006]. Samples were paraffin wax embedded, sectioned, and stained with haematoxylin and eosin according to standard protocols [Brown et al., 2005; EUMORPHIA Consortium, 2007].

Mouse Sperm Analysis

Five *iv*/*iv* C3H and five C3H male mice were sacrificed by cervical dislocation according to Schedule 1 of the UK Animals (Scientific Procedures) Act 1986. The vas deferens and cauda epididymus were dissected out and placed in synthetic human tubal fluid (HTF) under mineral oil (Sigma, Poole Dorset, UK). Motile spermatozoa were expressed from the vas deferens by lacerating the tissue with a 25-gauge hypodermic needle six times and then gently massaging the cauda epididymis with a pair of fine forceps. Samples were incubated for 1 hr at 37°C, 5% CO₂, allowing the sperm to disperse fully, then diluted 1:10 in HTF and loaded onto preformed 20 µl four-chamber capillary slides (Leja, Nieuw-Vennep, The Netherlands). Analysis was with IVOS (Integrated Visual Optical System for sperm analysis) sperm analyzer (Hamilton-Thorne Biosciences, Beverly, MA) previously optimized for mouse sperm. Each sample was analyzed three times, and five fields counted per reading. Samples of epididymus were also prepared for electron microscopy.

Table 1. Mouse Genotype and Group Characteristics

Genotype	<i>iv/iv</i>	<i>iv/iv</i>	<i>iv/iv</i>	<i>iv/iv</i>	<i>iv/iv</i>	<i>+/+</i>	<i>+/+</i>
Genetic background	Mixed	Mixed	Mixed	Mixed	C3H/HeH	C3H/HeH	C3H/HeH
Females			5	10	2	2	14
Males	5	5	5	9	3	3	6
Age (w) or range	5	9	15	31–42	14–23	13–25	11
Situs inversus	3/5	0/5	4/10	6/19	3/5	0/5	N/D
Rhinitis	5/5	5/5	10/10	19/19	5/5	0/5	0/20
Sinusitis	0/5	0/5	4/10	4/19	0/5	0/5	0/5
Otitis media	0/5	2/5	0/10	2/19	0/5	0/5	0/20
Lung histology	0/5 no abnormalities	0/5 no abnormalities	3/10 peri-bronchiolar lymphoid aggregates	1/19 peri-bronchiolar lymphoid aggregates	1/5 peri-bronchiolar lymphoid aggregates	1/5 peri-bronchiolar lymphoid aggregates	N/D

N/D, not done.

Table 2. Clinical Details with Local Reference Values

	Patient #730	Patient #616	“Classical PCD” <i>n</i> = 28 (%)	“Not PCD” <i>n</i> = 241 (%)
Age at test/diagnosis (years)	4	6	0–67.7 Mean 13.4; Median 5.3	0–67 Mean 16.4 Median 9.0 SD 19.4
Neonatal symptoms ^b	Yes	Yes	14 (50%)	
Persistent cough Persistent, wet cough	Yes	Yes	25 (89%) 23 (82%)	207 (86%) 152 (63%)
Persistent/frequent rhinitis	Yes	Yes	22 (79%)	128 (53%)
Sinusitis			5 (18%)	48 (20%)
Middle ear effusions	Yes	Yes	16 (57%)	42 (17%)
Situs inversus totalis	Situs solitus	Situs solitus	8 (29%)	10 (4%)
Heterotaxy ^a			3 (11%)	6 (2%)
Nasal NO (ppb)	49	24	<i>n</i> = 19 Range 2–70; Mean 33.7; SD 19.4	<i>n</i> = 59 Range 80–>2000 Mean 570 SD 350
Electron microscopy ODA ± IDA	Within normal limits ^d	Within normal limits ^d	25 (89%)	Within normal limits ^d [<i>n</i> = 169]
Radial spoke defect			3 (11%)	
Ciliary Function	Rapid, erratic, dyskinetic ciliary beating; CBF was immeasurable <i>n</i> = 2 assessments	Static cilia with occasional slow ciliary activity (mean CBF = 5.26 Hz SEM ± 0.63 by FFT analysis at one assessment) <i>n</i> = 3 assessments	24 static; 1 twitching; 3 dyskinetic	8.6–23.0° Mean 15.1; median 15.0 SD 2.5; all patterns within normal limits

The clinical characteristics of the index patients (#730 and #616), 28 consecutive patients seen in the Southampton PCD service diagnosed with “classical PCD” defined by abnormal ciliary function plus diagnostic ciliary TEM, and 241 consecutive patients (2006–2009) in whom PCD was excluded on the basis of light microscopy ± electron microscopy (“not PCD”).

^aHeterotaxy, left or right isomerism; complex cardiac disease.

ODA ± IDA: outer dynein arm defects ± inner dynein arm defects.

^bNeonatal pneumonia, admission to neonatal unit or respiratory distress at birth. Data from 26 patients.

^cEight patients (3.3%) had a mean CBF below 11 Hz, but this was deemed a secondary defect on the basis of other results.

^dIf defects fall below 60%, then the biopsy is considered nonPCD according to local reference data.

Patients

The research was approved by the Southampton and South West Hampshire Local Ethics Committee (LREC No 07/Q1702/109). Two patients with a clinical history consistent with PCD including abnormal ciliary function, but with normal axoneme ultrastructure, consented to genetic analysis. Clinical details are summarized in Table 2. Comparative data are given for consecutive “classical” PCD patients with ultrastructural anomalies and patients referred to the centre for PCD diagnostics who had the diagnosis excluded.

Ciliary Beat Analysis

Mouse tracheal rings were suspended in MEM (Invitrogen, Paisley, UK). For each sample, ciliary beat frequency (CBF) was determined at 37 °C at six independent points. Fast Fourier transform (FFT) analysis of a digitized photodiode signal from an Olympus inverted microscope (Olympus UK, KeyMed, Southend on Sea, UK) identified the dominant signal frequency. Human nasal epithelial cells were obtained from the inferior turbinate using disposable 2-mm Olympus EndoTherapy BC-202D cytology brushes (Olympus UK). Cells were resuspended in 1.5 ml supplemented BEGM

medium (CC3170 Clonetics, Lonza, Walkersville Inc., MD) at 37°C and 10 video microscopy sequences recorded at 250 frames per second (fps) using a FASTCAM high-speed video camera with a 100× oil immersion objective. CBF and beat pattern analysis were assessed by an experienced observer at 30 fps.

Electron Microscopy

Mouse and human samples were prepared following standard TEM and scanning electron microscopy (SEM) protocols [Bozzola and Russell, 1991; Rutland et al., 1982]. For each sample, 300 cilia cross-sections were examined at ×30,000 magnification; ultrastructural defects in dynein arms, microtubules or deviations from the normal 9+2 microtubule arrangement were counted by an expert observer.

Genetic Analysis

The 82 *DNAH11* exons were amplified with intronic primers as previously described [Bartoloni et al., 2002] and directly sequenced. Sequence variants were named according to NM_003777.3 cDNA

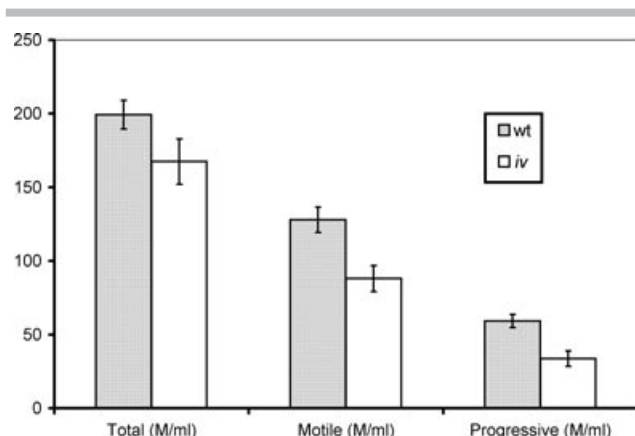


Figure 3. Mouse sperm motility. Analysis with IVOS sperm analyser optimized for mouse sperm showing sperm counts (million per ml $\pm$ SEM) for C3H wild-type (shaded bars) and C3H *iv/iv* mice. Each sample was analyzed three times, and five fields counted per reading giving total count of all sperm (total), motile sperm count (motile), and count of sperm with progressive motility (progressive).

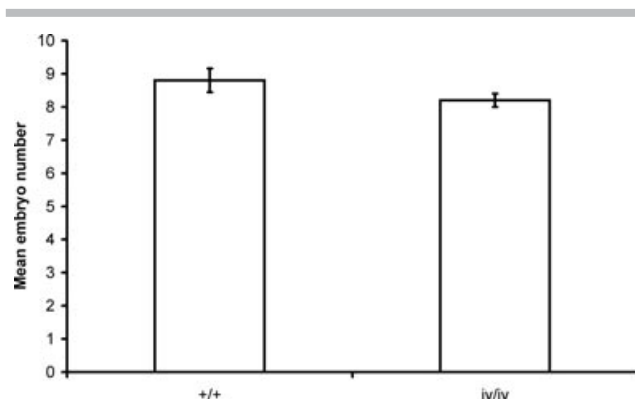


Figure 4. Mouse male fertility. The number of implanted embryos at 8.5 d.p.c. from C3H *iv/iv* and C3H wild-type sibling control males mated with C57Bl6/J wild-type females. Mean of 10 in each group $\pm$ SEM.

weaned from our homozygous *iv/iv* colony are reduced over controls (data not shown), raising the possibility of sperm-mediated fertility defects. However, a proportion of *iv/iv* embryos are known to die in utero, during mid or late gestation, due to situs-associated cardiac defects [Icardo, 1990; Icardo and Sanchez de Vega, 1991]. We therefore assessed fertility of *iv/iv* males by analyzing the number of implanted embryos at 8.5 d.p.c.; this is prior to cardiac-associated lethality. The analysis revealed 10 litters fathered by *iv/iv* males to give 82 implanted embryos, compared to 87 from wild-type control fathers (Fig. 4). Statistical analysis revealed no significant differences between the sample groups, showing the *iv/iv* males to be normally fertile.

iv Mice Demonstrate Chronic Catarrhal Rhinitis and Sinusitis

Consistent with previous reports [Hummel and Chapman, 1959; Layton, 1976], no external differences were observed between *iv/iv* and control mice. Necropsy analysis demonstrated randomized situs. In the *iv/iv* C3H mice collected for beat frequency analysis

8/16 were situs solitus, 7/16 situs inversus, and one situs ambiguus. From all *iv/iv* individuals on all backgrounds, 24/60 were situs inversus or situs ambiguus. Both gross and histological analysis of standard lung sections that include all lobes and major airways revealed no obvious pathology. In a minority of *iv/iv* and control mice more than 13 weeks old, anatomically normal peribronchiolar lymphoid aggregates were associated with the major airways (Table 1). In particular, there was no evidence of infection or inflammatory disease of the airways (including bronchiectasis), blood vessels, interstitium, or alveoli (data not shown). In contrast, histological analysis of the heads revealed catarrhal rhinitis, characterized by intranasal mucus in the caudal nasal passages variably admixed with neutrophil leukocytes, a phenotype that was fully penetrant in both male and female inbred *iv/iv* and *iv/iv* C3H mice (Table 1). Abnormal mucus accumulations in *iv/iv* mice were distinguishable from anatomically normal nasal mucus that consisted of thin ribbons of mucus on the surface of the brush border in wild type (WT) mice (Fig. 5A-I). Otitis media, another common symptom in PCD patients, was found in 9% of *iv/iv* mice and was invariably unilateral and characterized by serous effusion and mild thickening of the mucoperiosteum compared with C3H controls (Fig. 5M-P) and the normal contralateral ear (data not shown).

To assess the progressive nature of the PCD-related disease of *iv* mice, we analyzed the phenotype longitudinally in inbred *iv/iv* mice. Cohorts of five or more mice were analyzed at 5, 9, 15, and 31–42 weeks of age (Table 1). At 5 weeks, relatively small amounts of bland mucus or mucus containing small numbers of leukocytes were found in the caudal nasal passages of *iv/iv* mice (Fig. 5C and D). At 31–42 weeks of age, intranasal accumulations of mucus were extensive, admixed with viable and necrotic neutrophil leukocytes (Fig. 5E and F), and were more widely distributed into the mid and rostral snout (Fig. 5I and J). In contrast, sinusitis was not evident in either 5- or 9-week mice while 4/10 and 4/19 mice showed sinusitis at 15 and 31–42 weeks, respectively. (Fig. 5K and L Table 1). The earliest occurrence of otitis media (OM) was at 5 weeks and the incidence of Otitis media did not subsequently increase with age.

DNAH11 Mutations in Patients with PCD

The data from the mouse clearly demonstrate that *Dnahc11* mutations can result in immotile tracheal cilia leading to PCD-like phenotypes. Two atypical PCD patients with a clinical history highly suspicious for PCD (Table 2), abnormal ciliary function at 37°C, but normal ciliary ultrastructure were therefore investigated for *DNAH11* mutations. Both patients were white Caucasians, unrelated and consanguinity was unlikely. Patient #616 exhibited predominantly static nasal epithelial cilia at 37°C (confirmed on three separate occasions). Occasional slow ciliary activity was measured at one assessment with a mean CBF of 5.26 Hz (SEM = 0.63 Hz). In contrast, patient #730 demonstrated abnormally rapid and dyskinetic ciliary beating (confirmed on two separate occasions). The rapid dyskinetic cilia exhibited erratic interrupted “twitching” and neither performed rhythmical ciliary beating, nor completed a classical range of movement, preventing accurate CBF measurements. TEM analysis of nasal cilia showed no structural differences in either patient compared to normal (Fig. 6A-D), both showing numbers of structural defects within the range of non-PCD samples examined from the clinic (Table 2).

The clinical characteristics and diagnostic investigations are summarized in Table 2. For comparison, we present the clinical characteristics of 27 consecutive patients seen in the Southampton PCD

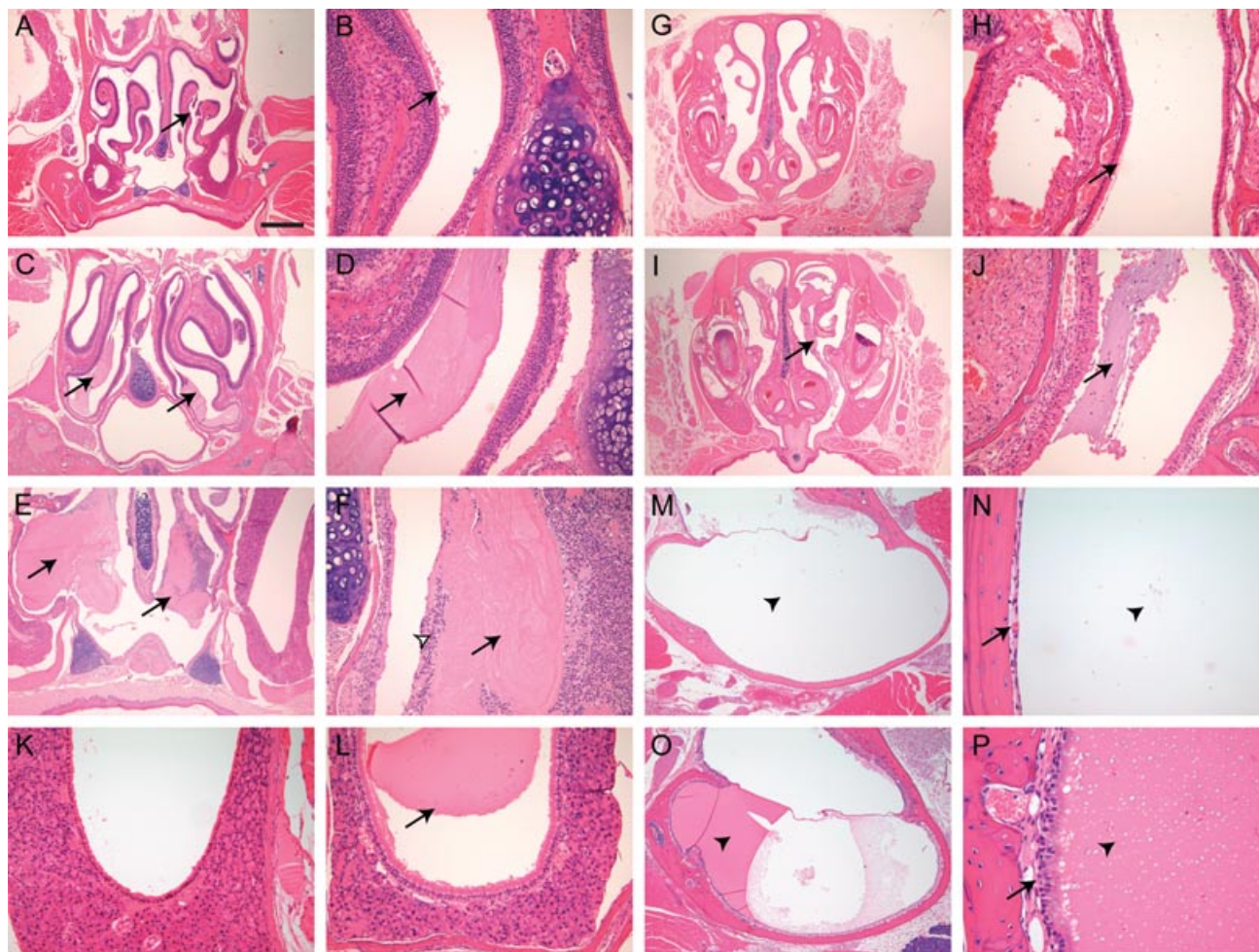


Figure 5. Catarrhal rhinitis, sinusitis, and otitis media in *iv/iv* mice. A and B: Caudal nasal passages of 44 week wild-type C3H female control; arrow indicates normal mucus; C and D: caudal nasal passages in 5 week *iv/iv* male; arrow indicates abnormal accumulations of bland mucus; E and F: caudal nasal passages in 39 week *iv/iv* female, arrow indicates increased accumulations of mucus (E), admixed with neutrophil leukocytes, indicated by open arrowhead (F); G and H: mid nasal passages in 44 week wild-type control, arrow indicates normal mucus. I and J: Mid nasal passages in 42 week *iv/iv* mouse, arrow indicates abnormal mucus and neutrophil leukocytes. K: normal air-filled sinus in a 44 week wild-type female control; L: sinus in a 41 week *iv/iv* female, arrow indicates serous effusion; M and N: middle ear in 44 week wild-type female, arrowhead indicates normal mucosa; O and P: otitis media in 41 week *iv/iv* female, arrowhead indicates serous fluid in bulla, arrow indicates slightly thickened mucosa. Scale bar shown in panel A corresponds to 500 μ m in panels A, C, E, G, I, M, O; 100 μ m in panels B, D, F, H, J, K, L; 50 μ m in panels N, P.

service diagnosed with “classical PCD”. This was defined by abnormal ciliary function plus diagnostic ciliary TEM. Details of 241 consecutive patients in whom PCD was excluded (“non-PCD”) are also included. PCD was excluded on the basis of normal ciliary function (assessed by high-speed video microscopy analysis) and when equivocal, confirmed as normal by further investigation including electron microscopy.

DNA from both patients was screened for mutations of *DNAH11*. Two sequence variations were detected in patient #730 (Fig. 7). A nonsense mutation in exon 53, c.8719C>T (g.21755845C>T NCBI36/hg18), changes arginine 2907 (in the AAA4 domain after the 4th P-loop) into a stop codon (p.Arg2907*). A missense mutation in exon 47, c.7793C>T (g.21744970C>T NCBI36/hg18), changes the highly conserved proline 2598 in the third AAA domain, to leucine (p.Pro2598Leu). This variation is not present in dbSNP or control samples and is identified as “probably damaging” by Polyphen (its highest score). In patient #616, only one mutation was detected (Fig. 7), a nonsense mutation in exon 39 (c.6527C>A,

g.21711640C>A NCBI36/hg18) changing serine 2176 into a stop codon (p.Ser2176*), positioned in AAA2, just before the second P-loop. Sanger sequencing of amplified exons does not detect heterozygous deletions, hence deletion of one or more exons cannot be ruled out.

Discussion

This study has identified the *iv* mouse as a viable model for aspects of the human disease PCD. This mouse line has been constantly bred as a homozygous colony for over 50 years, demonstrating that it is robust, viable, and that it demands only normal husbandry, in contrast to the viability issues seen in other models including *Dnahc5* [Tan et al., 2007], nm1054 [Lee et al., 2008] and the complex preparatory considerations associated with the *Dnaic* conditional mutant [Ostrowski et al., 2010]. We have not only confirmed that the *iv* model has static respiratory cilia at 37°C, but that it develops PCD-related disease including rhinitis, sinusitis, and otitis media.

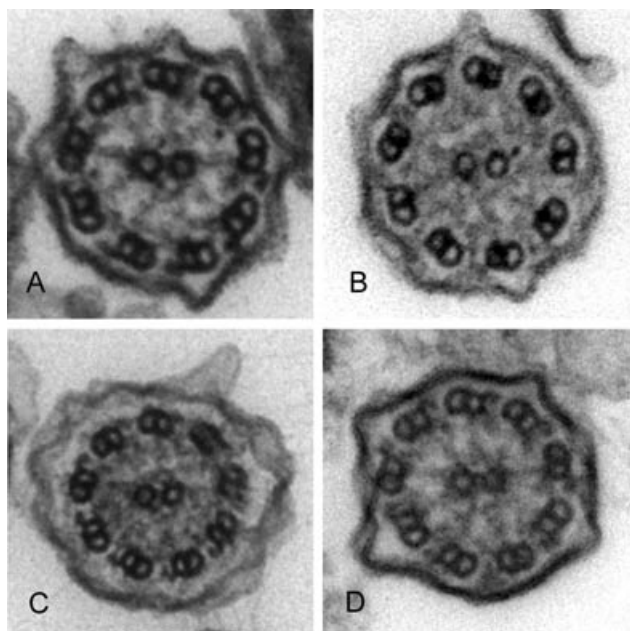


Figure 6. Cilia ultrastructure—human. Human cilia cross-sections examined by TEM. Normal (A) and cross-section from a “classical” PCD patient with an outer dynein arm (ODA) defect (B), cross-sections from patients #616 (C), and #730 (D) both carrying *DNAH11* mutations.

Consistent with other PCD mouse models [Ibanez-Tallon et al., 2002; Lee et al., 2008; Tanaka et al., 2004], none of the mice in this study developed significant lung pathology. This is perhaps an age-related phenomenon (our oldest mice were 42 weeks old), or lack of disease may be associated with the relatively sterile environment of the animal facilities as suggested for CF models [Davidson and Rolfe, 2001]. The mice were maintained specific pathogen free, so further work will be required to establish whether introduction of respiratory pathogens results in lower airway disease. Sinusitis and otitis media were seen in mouse mutants lacking the Wnt inhibitor *Cby1* (MIM# 607757) that were unable to clear bacteria from the sinuses upon intranasal bacterial challenge leading to upper airway inflammation, however additional morphological pulmonary defects were also evident from shortly after birth [Love et al., 2010]. Strikingly, lower airway disease, including pneumonia, was reported for *Spag6* (MIM# 605730), *Spag16* (MIM# 6121736) double mutants, however mortality is very high with 100% lethality by 5 weeks of age, making it far more severe than human PCD [Zhang et al., 2007]. It is tempting to speculate that it is the presence of chronic infection in man, taking place over a period of years rather than months, which is responsible for bronchiectasis and other lung pathology rather than a direct link to dyskinesia.

There was evidence of decreased progressive sperm motility in the *iv* mice, but since the line has been successfully bred from homozygotes for many years, any associated subfertility cannot be considered a major problem with this mutation, a conclusion supported by the results of the fertility test. Although male infertility is said to be common in PCD, it is not universal [Munro et al., 1994]. Indeed, one of the *DNAH11* PCD patients reported by Schwabe [Schwabe et al., 2008] had apparently fathered two children without medical assistance. This might suggest that this dynein is normally absent in sperm, although the conservative changes seen in mouse sperm motility argue that there is more likely to be additional functional redundancy in the sperm.

This study contributes to the evolving story concerning *DNAH11* as a cause of PCD. Mutations in *DNAH11* have to date been described in a family with PCD [Schwabe et al., 2008] associated with a sibling pair and in two isolated cases [Bartoloni et al., 2002; Piferi et al., 2010]. The static cilia in our mouse model, contrasting with previously described human PCD cases, led us to investigate a patient (#616) with consistently static cilia, normal ciliary ultrastructure, and a typical clinical PCD phenotype. A second patient (#730) with PCD phenotype, normal ciliary ultrastructure and stiff, hyperfrequent cilia had two mutations in *DNAH11*, consistent with this being the primary defect. We were only able to identify one mutation in patient #616, although both parents are asymptomatic and it is therefore unlikely to be a simple dominant mutation. The entire coding sequence, the 5' and 3' UTRs, and the intronic regions flanking each exon were ruled out. The promoter, which is not well described yet, and regulatory regions were not screened. A second *DNAH11* mutation resulting from deletion of one or more exons would not be evident from normal DNA sequencing and remains a possibility. Similarly allele dropout due to a mutation in the primer binding site cannot be completely excluded. The prospect that the patient carries mutations in another PCD gene must also be considered with the *DNAH11* mutation contributing to a multigenic defect in combination with one or more other PCD locus mutations. A recent report of a PCD patient with only a single frameshift mutation in *DNAH11* was suggested to result from digenic inheritance with a *DNAH2* (MIM# 603333) variant [Berg et al., 2011], although the authors indicate that further studies are required. However, these human data, combined with the evidence that mutations in the mouse homolog can be associated with static cilia, supports the hypothesis that *DNAH11* mutations can cause static cilia, in addition to the previously described association with stiff vibrating/hyperfrequent cilia. Mutations in this gene should therefore be considered in patients with a PCD phenotype associated with either static or hyperfrequent cilia.

The three *DNAH11* mutations that we have identified seem highly likely to affect protein function. Two are truncations resulting from premature stop codons and one is the substitution of a highly conserved nonhydrophobic proline with a hydrophobic leucine within the third AAA domain (p.2598Pro>Leu). Prior to this study, all mutations identified in *DNAH11* (Fig. 7) have been associated with hyperfrequent ciliary beating. Mutations in genes resulting in outer dynein arm (ODA) defects (as determined by TEM) generally have a static ciliary phenotype [Chilvers et al., 2003]. As ODAs are present in *DNAH11* and *Dnahc11* mutants (as assessed by TEM), it seems possible that this dynein may have an auxiliary or regulatory role, resulting in altered mechanical characteristics of the axoneme if the molecule is absent or substantially truncated. However, the basis of the immotile cilia in patient #616 remains difficult to ascertain as only a single truncated allele has so far been identified. In contrast, the *iv* and *lrm3* mouse lines show immotile tracheal cilia from presumed null mutations [Ermakov et al., 2009; Supp et al., 1999] arguing that the true null phenotype is immotile cilia. Further study will be required to determine whether mouse and human cilia truly have identical requirements for *DNAH11*/*Dnahc11* function.

A model of a human genetic disease can seek to recreate the mutation, the physiological defect, defects at the cellular level, or all of these. On occasions all outcomes will occur in combination, but this is not always the case. The *iv* mouse model clearly recreates the physiological phenotype of immotile respiratory cilia through a *Dnahc11* mutation. It therefore should be useful for modeling and therapy development associated with (1) loss of *DNAH11* function, (2) loss of respiratory cilia motility, and (3) loss of cilia-driven

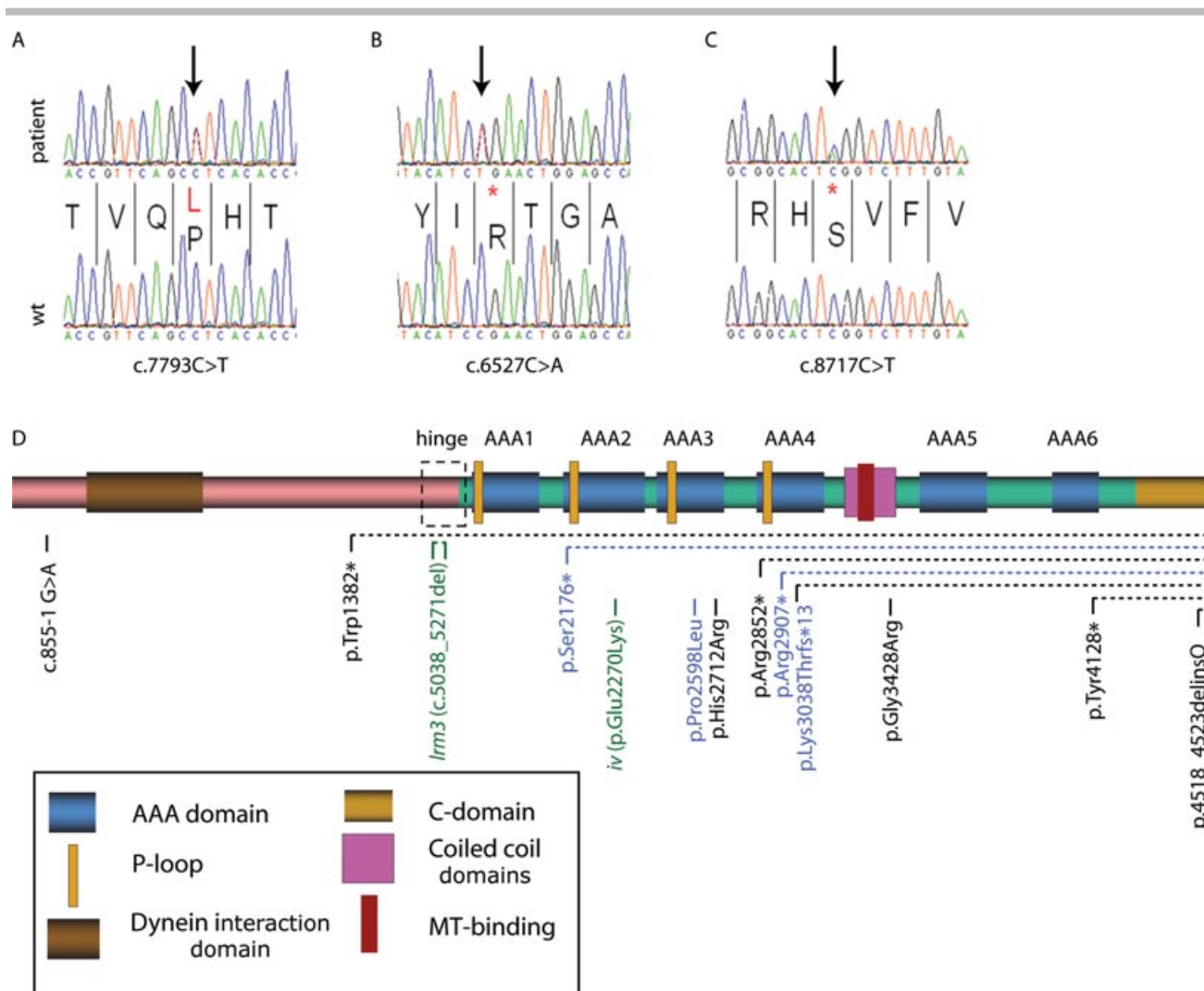


Figure 7. Novel mutations found in this study. A–C: Chromatograms of novel mutations found in this study. D: Schematic of DNAH11/Dnahc11 protein showing location of mutations from this study (blue) and previous publications from mouse (green) and human (black) relative to the recognized dynein heavy chain domains. Mutations causing truncation or frame shift (FS) are shown with a dotted line downstream of the mutation. From the N-terminus mutations are from the following studies c.855–1G>A [Pifferi et al., 2010]; p.Trp1382* [Pifferi et al., 2010]; Irm3 mouse–50 amino acids missing [Ermakov et al., 2009]; p.Ser2176* (this study); iv mouse [Supp et al., 1997]; p.Pro2598Leu (this study); p.His2712Arg [Pifferi et al., 2010]; p.Arg2852* [Bartoloni et al., 2002]; p.Arg2907* (this study); p.Lys3038Thrfs*13 [Berg et al., 2011]; p.Gly3428Arg [Pifferi et al., 2010]; p.Tyr4128* [Schwabe et al., 2008]; p.4518_4523delinsQ [Schwabe et al., 2008].

clearance from the lung. We have identified a viable and readily accessible mouse model of PCD that will allow future studies into therapeutic approaches including, but not limited to, gene therapy. It will allow the hypothesis that recurrent infection, rather than PCD itself, underlies bronchiectasis to be tested. Importantly, it will allow PCD and putative therapies to be investigated from birth into old age, something not possible with current models. Moreover, we have highlighted important implications for patient diagnosis. TEM is often quoted as the gold standard test in the diagnostic work-up of PCD. This study contributes to the growing evidence that PCD will be missed in a proportion of patients with normal ciliary architecture if ciliary function studies are not routinely performed. Indeed some studies have suggested that normal electron microscopy is as common as 10–15% in PCD. To date *DNAH11* is the only gene with mutations associated with PCD that has been described in patients with normal TEM and the *iv* mouse models this.

Acknowledgments

Our thanks to the patients and their families for their participation in this study. The staff of Biomedical Imaging Unit Southampton provided technical support and assistance. Adele Austin, Caroline Barker, Jenny Corrigan, Liz Darley at MRC Harwell for histology support. Staff in the Mary Lyon Centre for animal care. The Gordon Research Conference on Cilia, Mucus, and Mucociliary Interactions for introducing the authors to one another.

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