Mis-localization of DNAH5 and DNAH9 in respiratory cells from primary ciliary dyskinesia patients

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Online Data Supplement

Methods

Patients and families. Signed and informed consent was obtained from patients and family members using protocols approved by the Institutional Ethics Review Board at the University of Freiburg and collaborating institutions. With the help of the German and Swiss PCD support groups, we obtained trans-nasal brush biopsies from a large cohort of patients fulfilling diagnostic criteria of PCD. Six American PCD patients were also included. Two patients with cystic fibrosis, three with recurrent respiratory infections and 10 healthy volunteers were included as controls. Samples were evaluated in a blinded fashion to avoid investigator bias.

Generation of DNAH5-specific antibodies. Antibodies were raised against a 284 amino acid N-terminal fragment (amino acids 42-325) of DNAH5. The corresponding cDNA fragment was cloned into pGEX4-T1 (Amersham, Freiburg, Germany) and expressed as a GST fusion protein in *E.coli*. Purified protein was used to immunize New Zealand rabbits. Animal experiments for antibody production were granted by the local government. Antibodies were affinity column purified using a maltose binding protein (New England Biolabs, Frankfurt, Germany) fused to the N-terminal DNAH5 fragment.

Immunoblotting. A fresh pig trachea was obtained from a local butcher. Axonemes were prepared and proteins were extracted without photolysis according to previously published procedures (E1,E2). Proteins were separated on NuPAGE 3-8% tris-acetate gels (Invitrogen, Karlsruhe, Germany) and blotted onto PVDF membranes (Amersham). Blots were processed according to standard methods using a 1:1000 dilution of DNAH5 antibody and a 1:2500 dilution of HRP conjugated anti-rabbit secondary antibodies (Santa Cruz, Heidelberg, Germany). Bands were visualized with ECL plus (Amersham).

Immuno fluorescence analysis. Respiratory epithelial cells were obtained by trans-nasal brush biopsy using a cytobrush plus (Medscand Malmö, Sweden) and suspended in cell culture medium. Sperm cells were washed with phosphate buffered saline (PBS) to remove seminal plasma. Samples were spread onto glass slides, air dried and stored at -80°C until use. IF staining was performed according to standard protocols. Briefly, cells were rinsed with PBS, fixed with 4% paraformaldehyde and treated with 0.2% Triton-X 100 prior to blocking with 0.5% skim milk. All reagents were dissolved in PBS and antibodies were diluted in 0.5% skim milk. Cells were covered with primary antibodies for at least 2 hours and with secondary antibody for 30 minutes at room temperature. Samples were washed 2-4 times with PBS after each step. Appropriate controls were performed omitting the primary antibodies. Commercially available primary antibodies were: mouse-anti-acetylated-α-tubulin, mouse-anti-γ-tubulin (Sigma, Taufkirchen, Gernmany) and mouse-anti-DNAH9 (BD Biosciences, Heidelberg, Germany). Anti-DNAH9 antibodies have been previously described (E1). We confirmed the specificity by Western blot, which demonstrated a specific dynein heavy chain band distinct from the DNAH5 band. Appropriate secondary antibodies (Alexa Fluor 488, Alexa Fluor 546) were from Molecular Probes (Invitrogen). DNA was stained with 10 µg/ml Hoechst 33342 (Sigma) in H_2O. Images were taken on a Zeiss laser scan confocal microscope (LSM 510 iUV).

High-speed video analysis of ciliary beat frequency. Ciliary beat frequency was assessed with the SAVA system (Sisson-Ammons Video Analysis of ciliary beat frequency) (E3). Trans nasal brush biopsies were rinsed in cell culture medium and immediately viewed with an Olympus IMT-2 inverted phase-contrast microscope equipped with a Redlake ES-310Turbo monochrome high-speed video camera (Redlake, San Diego, USA) and a 40x objective. Digital image sampling was

performed at 125 frames per second and 640x480 pixel resolution. The ciliary beat pattern was evaluated on slow motion playbacks.

References

E1. Reed W, Carson JL, Moats-Staats BM, Lucier T, Hu P, Brighton L, Gambling TM, Huang CH, Leigh MW, Collier AM. Characterization of an axonemal dynein heavy chain expressed early in airway epithelial ciliogenesis. *Am J Respir Cell Mol Biol* 2000;23: 734-741.

E2. Hastie AT. Isolation of respiratory cilia. *Methods Cell Biol* 1995;47:93-98.

E3. Sisson JH, Stoner JA, Ammons BA, Wyatt TA. All-digital image capture and whole-field analysis of ciliary beat frequency. *J Microsc* 2003;211:103-111.

Web-Supplementary videos:

- respiratory_cilia_normal_function
- F651_respiratory_cilia_dyskinesia
- F649II2_respiratory_cilia_immotility
- F373II3_sperm_immotiliy
- sperm_normal_function