

Cholangiocyte cilia are abnormal in syndromic and non-syndromic biliary atresia

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Biliary atresia (BA) is a neonatal disorder characterized by aggressive fibroinflammatory obliteration of the biliary tract. Approximately 20 percent of BA patients demonstrate left–right laterality defects (syndromic BA). Cilia participate in important physiological functions in cholangiocytes, and as some ciliopathies have been associated with both laterality defects and hepatic fibrosis, we hypothesized that patients with syndromic BA exhibit abnormalities of cholangiocyte cilia that disrupt cholangiocyte homeostasis. Nine BA specimens were studied, including pre-Kasai diagnostic biopsies ($n=7$) and liver explants ($n=2$). Five specimens were from patients with laterality defects. These were compared with normal pediatric livers, as well as livers affected by primary sclerosing cholangitis, Wilson's disease, and cardiac cirrhosis. Biopsy sections were stained with antibodies against keratin 19 (a cholangiocyte marker) and acetylated α -tubulin (a cilia marker) and were visualized by confocal microscopy. Computer-assisted relative quantification was used to compare staining of cilia within bile ducts among samples. Surprisingly, cilia in BA specimens were significantly shorter, abnormal in their orientation, and less abundant compared with normal liver and disease controls regardless of the presence of a laterality defect. There are significant abnormalities of cholangiocyte cilia in both syndromic and non-syndromic BA livers compared with normal livers and livers affected by other cholestatic diseases. Although this may result from severe cholestasis or inflammation, it may also reflect common mechanistic pathways in different forms of BA and may have important implications for understanding the progression of the disease.

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Biliary atresia (BA) is a neonatal disease with an incidence of 1 in 8000–18000 live births that is characterized by aggressive fibroinflammatory obliteration of the extrahepatic biliary tract.¹ The etiology of BA is unknown but may involve environmental, infectious, and genetic factors. Although most BA patients undergo a Kasai portoenterostomy at the time of diagnosis to facilitate bile drainage, 70 to 80 percent of patients ultimately require transplant, and the disease is the most

common indication for liver transplantation in the pediatric population.^{1–3}

Approximately 20 percent of patients with BA have a variant form called syndromic BA (also known as embryonic BA or BA with splenic malformation syndrome) and demonstrate laterality defects, including malrotation, dextrocardia, and polysplenia.⁴ This suggests that a ciliary defect might be involved in the pathophysiology of BA, at least in this subset of patients, as abnormal function of primary cilia during embryogenesis has been implicated in the development of laterality defects.^{1,5} Primary cilia are thin, solitary tubular projections present on the cell membranes of most epithelial and stromal cells. In the liver, cholangiocytes but not hepatocytes possess cilia.⁶ Cholangiocyte cilia have been well studied and found to have various functions, including mechanotransduction

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of luminal flow; detection of biliary nucleotides, bile acids, and luminal tonicity; and interactions with biliary exosomes.^{6–11}

The classic cholangiocyte ciliopathy is autosomal recessive polycystic kidney disease, which is associated with congenital hepatic fibrosis. Other ciliopathies associated with liver fibrosis include Meckel syndrome, nephronophthisis, and Joubert syndrome.^{12–14} The *inv* mutant mouse, which possesses a defect in *inversin* (which encodes a ciliary basal body protein that regulates switching between canonical and non-canonical Wnt pathways), develops situs inversus and obstructive cholestasis.^{15–17} Ciliary abnormalities in BA have not been well studied. There have been only two case reports of patients with immotile cilia syndrome who also developed BA;^{18,19} however, a recent study by Hartley *et al*²⁰ found that the primary cilia-associated protein fibrocystin/polyductin is absent in the cholangiocytes of patients with BA, suggesting that ciliary defects in this disease might be more widespread.

We hypothesized that patients with syndromic BA would demonstrate ciliary defects and that this finding might differentiate syndromic from non-syndromic BA. We therefore undertook to examine cholangiocyte cilia in biopsies from both forms of BA livers and to compare them with biopsies from patients with normal livers or other forms of cholestatic liver disease.

Materials and methods

Human Tissues

Fixed liver tissue was obtained from the surgical pathology archives at the Children's Hospital of Philadelphia with the approval of the Institutional Review Board. BA samples were either from diagnostic biopsies or from liver explant tissue. For controls, we examined three histologically normal liver samples from children (two obtained from resected tissue adjacent to hepatoblastoma and one from the explant of a patient with metabolic disease) and three cholestatic disease controls (primary sclerosing cholangitis, Wilson's disease, and cardiac cirrhosis; Table 1). Tissues were fixed in 10% neutral-buffered formalin, dehydrated through serial alcohol washes using an automated processor, and cleared with xylene before embedding with paraffin.

Immunofluorescence Microscopy

Four-micron sections were dewaxed in xylene, rehydrated through serial ethanol washes, and subjected to microwave antigen retrieval in 10 mmol/l citric acid solution (pH 6.0). Sections were blocked with 1% bovine serum albumin in 0.1% Triton X-100/1x PBS and incubated with

primary antibodies at 4°C overnight: rabbit anti-cytokeratin 19 (K19; 1:100; Abcam, Cambridge, MA, USA), rabbit anti-Ki67 (1:100; Abcam), and mouse anti-acetylated α -tubulin (1:5000; Sigma, St Louis, MO, USA). Slides were incubated with the appropriate Cy2- or Cy3- (1:600) secondary antibodies (Jackson ImmunoResearch, West Grove, PA, USA) for 2 h at room temperature. For conventional immunofluorescence, images were captured using a Nikon E600 microscope (Nikon, Melville, NY, USA) equipped with a QICAM CCD camera (QImaging, Burnaby, BC, Canada) and processed using iVision software (BioVision Technologies, Exton, PA, USA). For confocal imaging, single slice and Z-stack images were captured using a Zeiss LSM-510 Meta confocal microscope (Thornwood, NY, USA).

Cell Counting, Computer-Assisted 3-D Reconstructions, and Relative Quantification of Cholangiocyte Cilia

Cell counting was performed manually with the assistance of ImageJ software (NIH, Bethesda, MD, USA). 3-D reconstructions of confocal immunofluorescence image Z-stacks were generated using the LSM Image Viewer (Carl Zeiss MicroImaging, Munich, Germany). To compare cholangiocyte cilia, we performed computer-assisted relative quantification by generating the ratio of intraluminal ciliary staining to the number of directly adjacent cholangiocyte nuclei in regions of interest. Only ducts with clear lumens were examined, and 3-D reconstructions were used to generate maximal information about the cilia projecting into a given duct. For any given area that we assessed, two confocal slices separated by at least 1.2 microns were taken from the same Z-stack for evaluation. Quantification of intraluminal biliary cilia staining was performed using iVision software (Biovision Technologies, Exton, PA, USA), and cholangiocyte nuclei were counted manually using ImageJ.

Results

We assessed nine BA specimens, five from patients with laterality defects (Table 1). BA samples were compared with three histologically normal pediatric livers and tissue from patients with cirrhosis resulting from other cholestatic liver diseases, including primary sclerosing cholangitis, Wilson's disease, or cardiac cirrhosis.

All livers were stained with antibodies against K19, to identify bile ducts, and against acetylated α -tubulin, to identify cilia. Histologically normal control livers demonstrated abundant intraluminal biliary cilia that projected into the lumen (Figure 1a, Supplementary Figure 1, Supplementary Movie 1). In the livers of the cholestatic disease controls

Table 1 Sources of tissue

Specimen	Pathology	Biopsy type	Age
<i>Control samples</i>			
C1 ^a	Normal tissue adjacent to hepatoblastoma	Resection	NA
C2 ^a	Normal tissue adjacent to hepatoblastoma	Resection	5 yrs
C3 ^a	Citrullinemia	Explant	6 m 22 d
C4	PSC	Explant	19 yrs
C5	Cardiac cirrhosis	Explant	9 yrs
C6	Wilson's	Explant	17 yrs

Specimen	Pathology	Biopsy type	Age	Sex	Race	Transplant by 2 yrs
<i>BA samples</i>						
B1	BA	Wedge	4 m 18 d	F	Black	No
B2	BA	Needle	2 m 20 d	M	Caucasian	No
B3	BA	Wedge	48 d	F	Caucasian	No
B4	BA	Needle	3 m 2 d	F	Black	No
B5	BASM	Wedge	27 d	F	Caucasian	No
B6	BASM	Explant	5 m 26 d	F	Caucasian	Yes
B7	BASM	Wedge	20 d	M	Black	No
B8a	BASM	Wedge	33 d	M	Hispanic	Yes (see B8b)
B8b ^b	"	Explant	6 m 5 d	"	"	"

Abbreviations: NA, not available; PSC, primary sclerosing cholangitis; BA, biliary atresia; BASM, biliary atresia with splenic malformation syndrome (also called syndromic BA).

^aHistologically normal.

^bB8a and B8b were from the same patient, taken at different times.

(Figures 1b–d, Supplementary Movies 2 and 3), cilia appeared to be relatively preserved when compared with normal tissues, although there was an increased degree of heterogeneity seen in these sections compared with the normal samples.

In contrast, the cilia in livers from patients with both the syndromic and non-syndromic forms of BA showed significant abnormalities when compared with normal and cholestatic disease controls. As demonstrated by single plane images and 3–D z-stack reconstructions (Figure 2, Supplementary Figures 2 and 3, Supplementary Movies 4 and 5), cilia in BA specimens were noticeably shorter, fewer in number, and abnormal in their angulation compared with normal liver and with the alternate disease controls. These differences were seen regardless of the source of the tissue (biopsy vs explant) or the presence of laterality defects. Computer-assisted relative quantification of these samples confirmed a statistically significant reduction in the ratio of cilia to cholangiocyte nuclei in all BA specimens compared with controls (Figure 3). We did not detect a difference in this ratio between BA and syndromic BA specimens.

As one possible explanation for the decreased cilia we observed is resorption in the context of mitosis, we estimated cholangiocyte proliferation using the proliferation marker Ki67 (Figure 4). As expected, in control livers, biliary Ki67 labeling was low (average of 1.4% of all cholangiocytes; raw count = 3/157; $n = 2$). There was a significant overall increase of biliary Ki67 labeling in BA (average of

6.2%, $P = 0.17$ compared with controls; raw count = 63/818; $n = 2$) and BASM (average of 9.6%, $P < 0.05$ compared with controls; raw count = 71/844; $n = 5$) specimens compared with normal controls.

Discussion

Here we report histological evidence of abnormalities in cholangiocyte cilia in BA livers compared with normal livers and livers affected by other cholestatic diseases. These changes included reduced numbers, shortened length, and abnormal orientation. Surprisingly, there were no detectable histological differences in the cilia from livers of patients with syndromic compared with non-syndromic BA.

Cilia fall into three broad categories. *Motile cilia* have a core formed from nine outer pairs of microtubule rods that encircle a single inner pair of microtubules (the '9 + 2' pattern). Dynein arms on the outer rods function as molecular motors that actively propel cilia in a whip-like manner.²¹ Case reports linking syndromic BA to abnormal cilia refer to motile cilia.^{18,19} *Nodal cilia* are located on cells of the embryonic node in vertebrates during development. They lack the two inner microtubule rods ('9 + 0' pattern) but possess rotational movement that is important in establishing morphogen gradients that determine left–right laterality.⁵ No link, however, between syndromic BA and defects in nodal

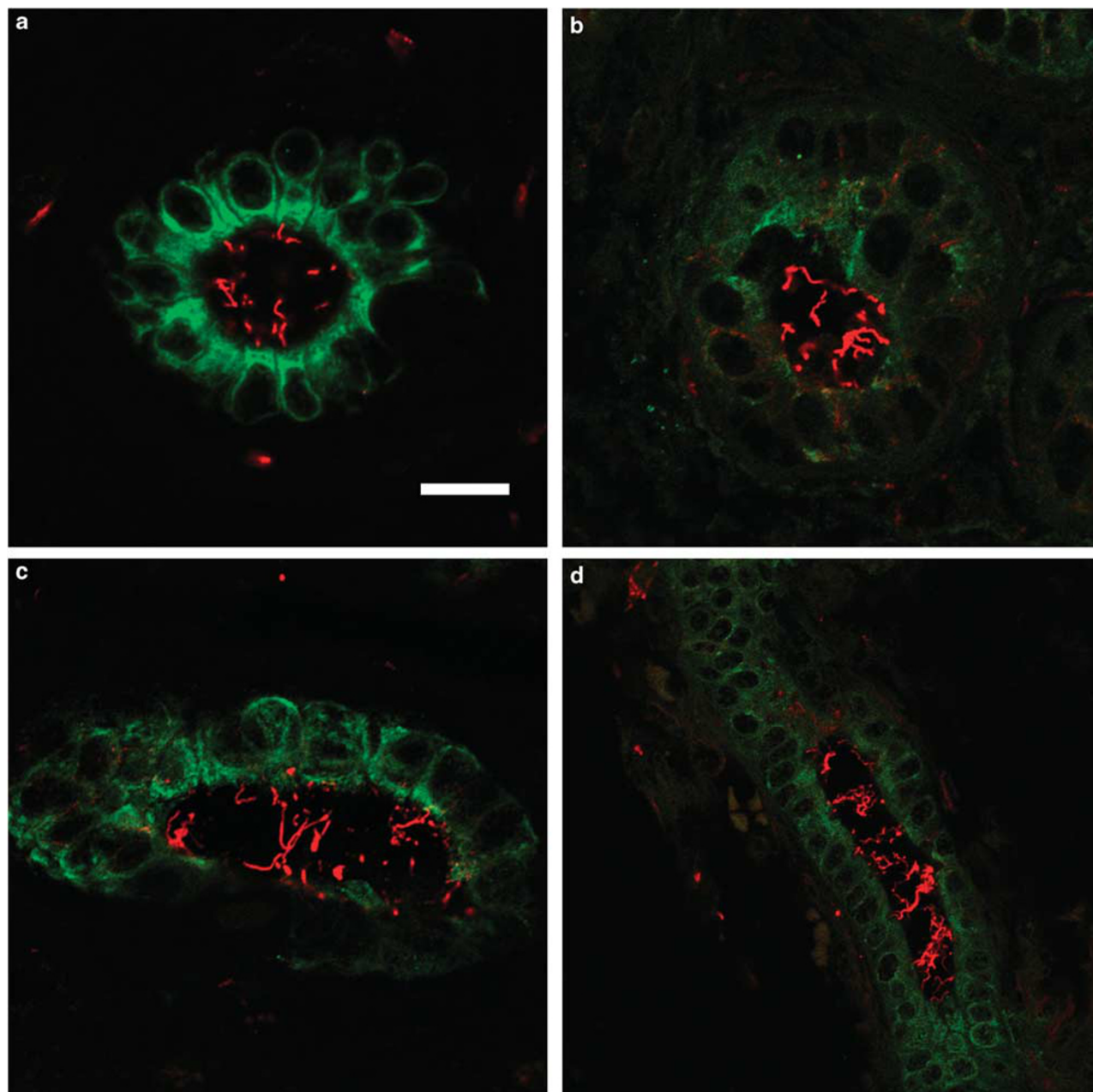


Figure 1 Confocal immunofluorescence microscopy of control livers. Green (Cy2) = K19 (bile ducts), red (Cy3) = acetylated α -tubulin (cilia). Note the quantity, length, and orientation of the intraluminal cilia. (a) Specimen C1, normal liver adjacent to hepatoblastoma; (b) specimen C4, primary sclerosing cholangitis; (c) specimen C5, cardiac cirrhosis; (d) specimen C6, Wilson's disease. Scale bar = 20 μ m in panels a–c, 10 μ m in panel d.

cilia has ever been identified. *Primary cilia*, the cilia found on cholangiocytes, also follow a 9 + 0 pattern but are not motile. These cilia are abnormal in ciliopathies such as polycystic kidney disease and were found to be abnormal in BA livers in this study.

There are several potential causes of abnormal cilia in BA. BA is characterized by aggressive portal inflammation, which could inflict ciliary injury. The severe chronic cholestasis seen in BA is associated with accumulation of cytotoxic hydrophobic bile acids, which damage mitochondria and cell mem-

branes, potentially including ciliary membranes.^{22,23} In addition, the basal bodies that anchor cilia are recycled to form the centrosomes of the spindle apparatus when cells enter the cell cycle.²⁴ Some of the decrease in intraluminal cilia we observed may reflect increased numbers of cholangiocytes undergoing mitosis in BA livers. Cholangiocyte proliferation is unlikely to fully explain the distortion in ciliary shape and angulation, however, and the relatively modest rise in Ki67 leaves open the possibility that other mechanisms are involved,

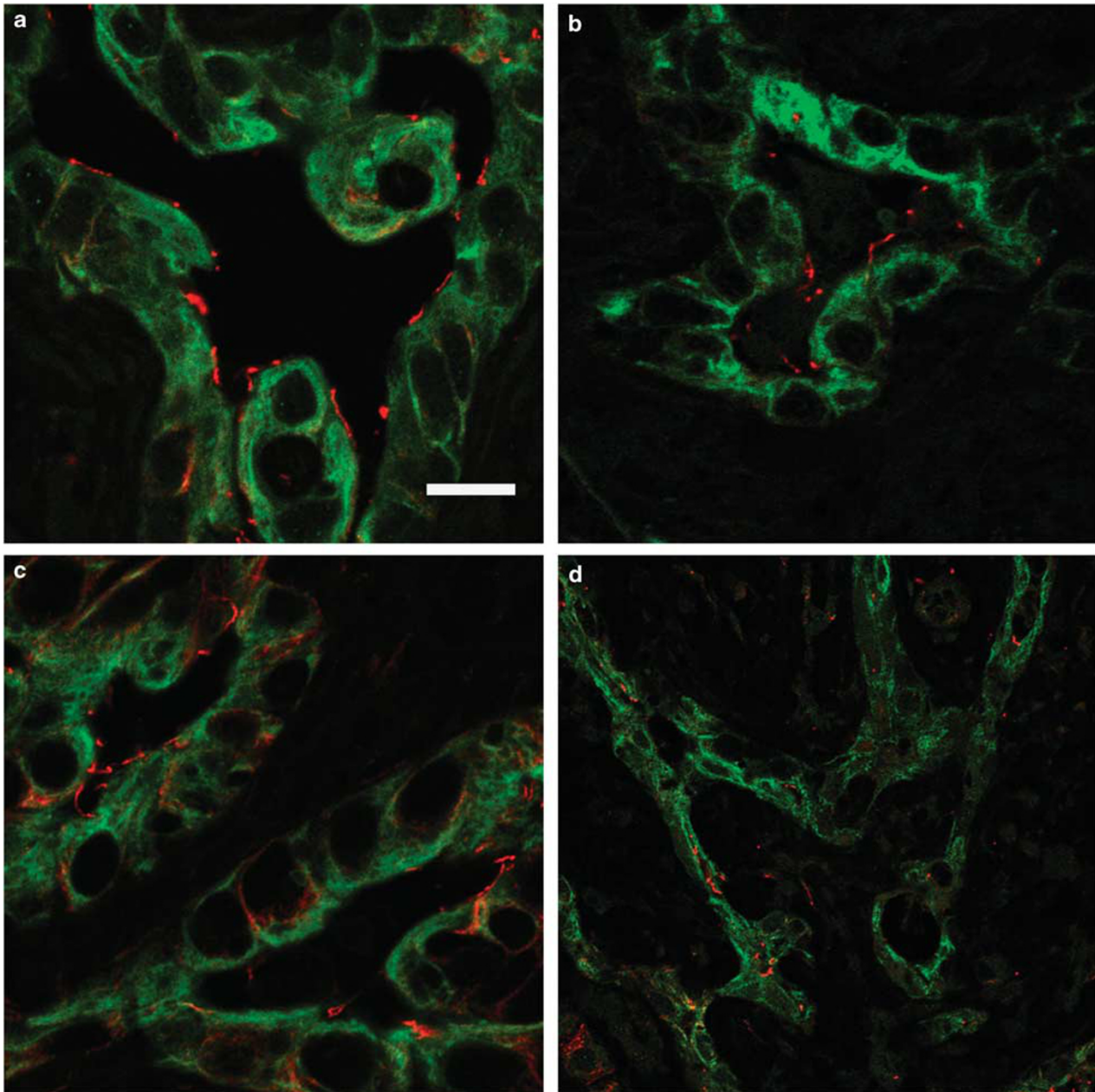


Figure 2 Confocal immunofluorescence microscopy of biliary atresia livers. Green (Cy2)=K19 (bile ducts), red (Cy3)=acetylated α -tubulin (cilia). Note the differences in quantity, length, and orientation of the intraluminal cilia compared with Figure 1. (a) Specimen B5, diagnostic wedge biopsy from patient with syndromic biliary atresia; (b) specimen B6, explant from patient with syndromic biliary atresia; (c) specimen B1, wedge biopsy from patient with biliary atresia; (d) specimen B2, diagnostic needle biopsy from patient with biliary atresia. Scale bar = 20 μ m in panels a–c, 10 μ m in panel d.

such as structural damage during the course of inflammation or damage due to the as yet unknown primary insult.

Abnormal cilia may also be relevant to the pathophysiology of BA. Overgaard *et al*²⁵ showed that *in vitro* stress-induced deciliation of MDCK II cells causes tight junction remodeling and reduced cell polarity. Masyuk *et al*⁷ demonstrated that chemical deciliation of cholangiocytes results in abnormal ciliary mechanotransduction, with resulting reductions in intracellular calcium, whereas Gradi-

lone *et al*²⁶ demonstrated increased cholangiocyte proliferation. Deciliation blunts the chemosensory function of cilia, leading to abnormal interactions with extracellular ADP, increased intracellular cyclic AMP levels, and increased cholangiocyte proliferation.^{9,27} Normal cilia appear to be important for graded cellular responses to sonic Hedgehog, which cholangiocytes both secrete and respond to during injury and which is pro-fibrogenic.^{28,29}

Our study is limited in that the samples we examined were all from advanced BA. Although

logistically difficult, it would be interesting to examine early BA livers for similar abnormalities. In addition, we relied on immunostaining of cilia, which does not detect ultrastructural defects; scanning and transmission electron microscopy of cilia

in early BA samples might clarify the nature of the cilia abnormalities we observed.

This is the first report of pervasive structural abnormalities in cholangiocyte primary cilia in BA. The lack of distinction between syndromic and non-syndromic BA was particularly surprising and suggests that there may be common damage pathways in the two forms of the disease. Whether the morphologically abnormal cilia we observe are functionally relevant to the course of the disease has yet to be determined.

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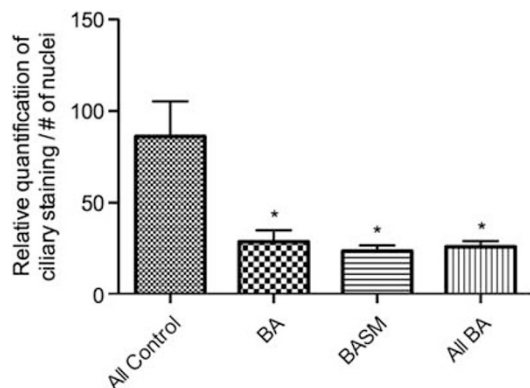


Figure 3 Relative quantification of intraductal cholangiocyte cilia staining. For each sample, intraductal anti-acetylated α -tubulin staining was quantified and divided by the number of associated cholangiocyte nuclei to generate a ratio for comparison among samples. Quantification was performed at two different levels within the Z-stack for each specimen. * $P < 0.05$ compared with control.

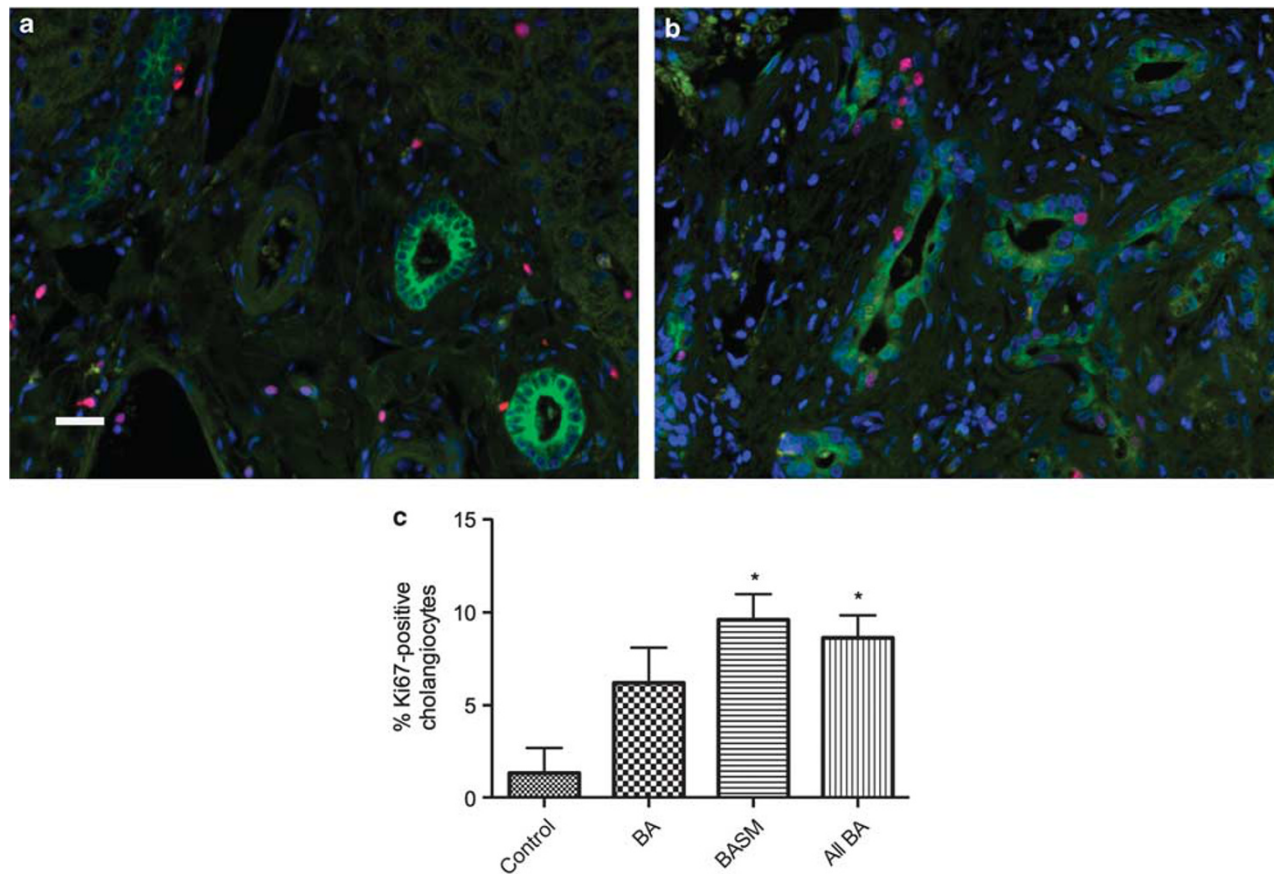


Figure 4 Cholangiocyte proliferation by Ki67 immunofluorescence microscopy. Green (Cy2)=K19 (bile ducts), red (Cy3)=Ki67 (proliferation marker). (a) Specimen C2 (normal); (b) specimen B6 (biliary atresia); (c) graph comparing average percentages of Ki67-positive cholangiocytes. * $P < 0.05$ compared with control. Scale bar = 20 μ m.

Disclosure/conflict of interest

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Supplementary Information accompanies the paper on Modern Pathology website (<http://www.nature.com/modpathol>)