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

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### Abstract

Biliary atresia is a neonatal disorder characterized by aggressive fibroinflammatory obliteration of the biliary tract. Approximately 20 percent of biliary atresia patients demonstrate left-right laterality defects (syndromic biliary atresia). Cilia participate in important physiologic functions in cholangiocytes, and since some ciliopathies have been associated with both laterality defects and hepatic fibrosis, we hypothesized that patients with syndromic biliary atresia exhibit abnormalities of cholangiocyte cilia that disrupt cholangiocyte homeostasis. Nine biliary atresia 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 to 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  $\alpha$ -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 biliary atresia specimens were significantly shorter, abnormal in their orientation, and less abundant compared to normal liver and disease controls regardless of the presence of a laterality defect.

**Conclusion**—There are significant abnormalities of cholangiocyte cilia in both syndromic and non-syndromic biliary atresia livers compared to normal livers and livers affected by other

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Supplementary information is available at *Modern Pathology*'s website.

### Disclosure/Conflict of Interest

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cholestatic diseases. While this may result from severe cholestasis or inflammation, it may also reflect common mechanistic pathways in different forms of biliary atresia and may have important implications for understanding the progression of the disease.

## Keywords

liver fibrosis; ciliopathy; laterality; situs inversus; cholestasis

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## Introduction

Biliary atresia is a neonatal disease with an incidence of 1 in 8,000–18,000 live births that is characterized by aggressive fibroinflammatory obliteration of the extrahepatic biliary tract.<sup>1</sup> The etiology of biliary atresia is unknown but may involve environmental, infectious, and genetic factors. Although most biliary atresia 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.<sup>1–3</sup>

Approximately 20 percent of patients with BA have a variant form called syndromic biliary atresia (also known as embryonic biliary atresia or biliary atresia with splenic malformation syndrome) and demonstrate laterality defects including malrotation, dextrocardia, and polysplenia.<sup>4</sup> This suggests that a ciliary defect might be involved in the pathophysiology of biliary atresia, at least in this subset of patients, since abnormal function of primary cilia during embryogenesis has been implicated in the development of laterality defects.<sup>1,5</sup> 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.<sup>6</sup> Cholangiocyte cilia have been well studied and found to have functions including mechanotransduction of luminal flow; detection of biliary nucleotides, bile acids, and luminal tonicity; and interactions with biliary exosomes.<sup>6–11</sup>

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.<sup>12–14</sup> 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.<sup>15–17</sup> Ciliary abnormalities in biliary atresia have not been well studied. There have been only two case reports of patients with immotile cilia syndrome who also developed biliary atresia;<sup>18,19</sup> however, a recent study by Hartley et al. found that the primary cilia-associated protein fibrocystin/polyductin is absent in the cholangiocytes of patients with biliary atresia, suggesting that ciliary defects in this disease might be more widespread.<sup>20</sup>

We hypothesized that patients with syndromic biliary atresia would demonstrate ciliary defects and that this finding might differentiate syndromic from non-syndromic biliary atresia. We therefore undertook to examine cholangiocyte cilia in biopsies from both forms of biliary atresia livers and to compare them to 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. Biliary atresia 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) as well as 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), rabbit anti-Ki67 (1:100; Abcam), and mouse anti-acetylated  $\alpha$ -tubulin (1:5000; Sigma, St. Louis, MO). Slides were incubated with the appropriate Cy2- or Cy3- (1:600) secondary antibodies (Jackson ImmunoResearch, West Grove, PA) for 2 hours at room temperature. For conventional immunofluorescence, images were captured using a Nikon E600 microscope (Nikon, Melville, NY) equipped with a QICAM CCD camera (QImaging, Burnaby, BC, Canada) and processed using iVision software (BioVision Technologies, Exton, PA). For confocal imaging, single slice and Z-stack images were captured using a Zeiss LSM-510 Meta confocal microscope (Thornwood, NY).

### 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). 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), and cholangiocyte nuclei were counted manually using ImageJ.

## Results

We assessed nine biliary atresia specimens, five from patients with laterality defects (Table 1). Biliary atresia samples were compared to three histologically normal pediatric livers as well as 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  $\alpha$ -tubulin, to identify cilia. Histologically normal control livers demonstrated abundant intraluminal biliary cilia that projected into the lumen (Fig. 1a, Suppl. Fig. 1, Suppl. Movie 1). In the livers of the cholestatic disease controls (Figs. 1b-d, Suppl. Movies 2 and 3), cilia appeared to be relatively preserved when compared to normal tissues, although there was an increased degree of heterogeneity seen in these sections compared to the normal samples.

In contrast, the cilia in livers from patients with both the syndromic and non-syndromic forms of biliary atresia showed significant abnormalities when compared to normal and cholestatic disease controls. As demonstrated by single plane images and 3-D z-stack reconstructions (Fig. 2, Suppl. Figs. 2 and 3, Suppl. Movies 4 and 5), cilia in biliary atresia 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 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 biliary atresia specimens compared with controls (Fig. 3). We did not detect a difference in this ratio between biliary atresia and syndromic biliary atresia specimens.

Since 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 (Fig. 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 biliary atresia (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 histologic evidence of abnormalities in cholangiocyte cilia in BA livers compared to normal livers and livers affected by other cholestatic diseases. These changes included reduced numbers, shortened length, and abnormal orientation. Surprisingly, there were no detectable histologic differences in cilia from livers of patients with syndromic compared to 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 act as molecular motors that actively propel cilia in a whip-like manner.<sup>21</sup> Case reports linking syndromic biliary atresia to abnormal cilia refer to



## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

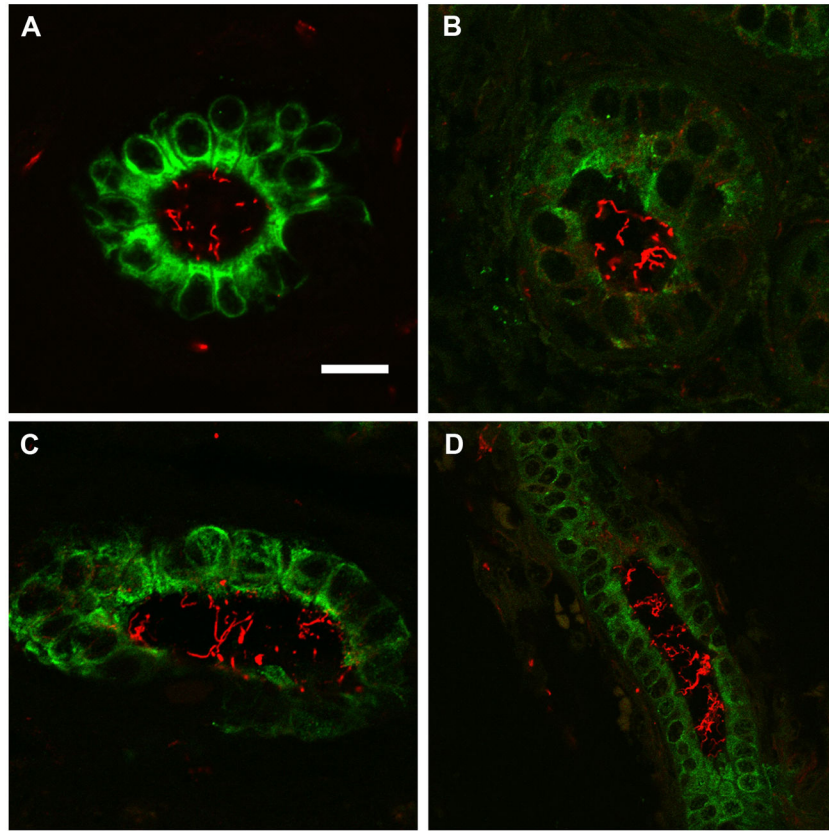
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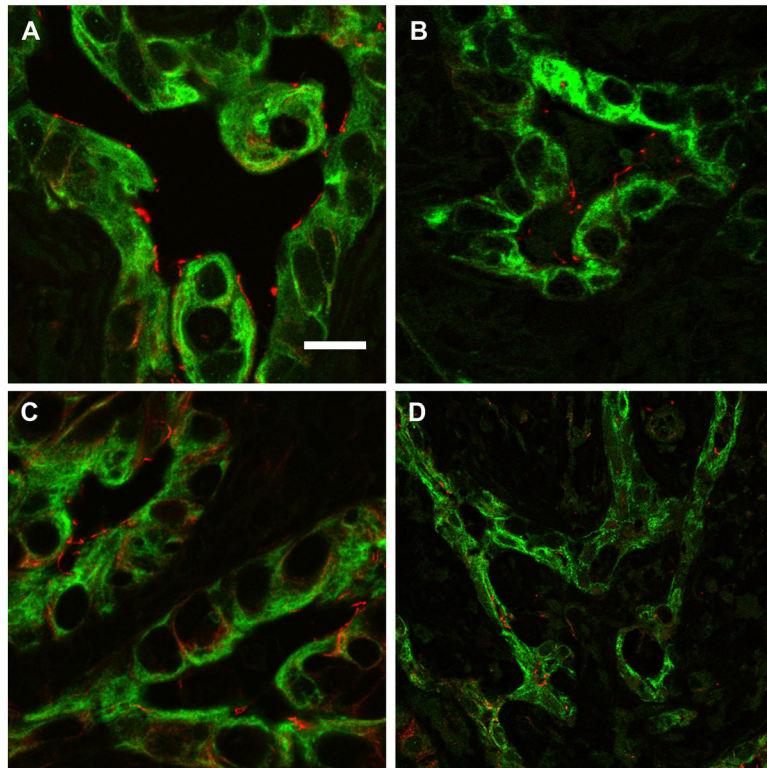
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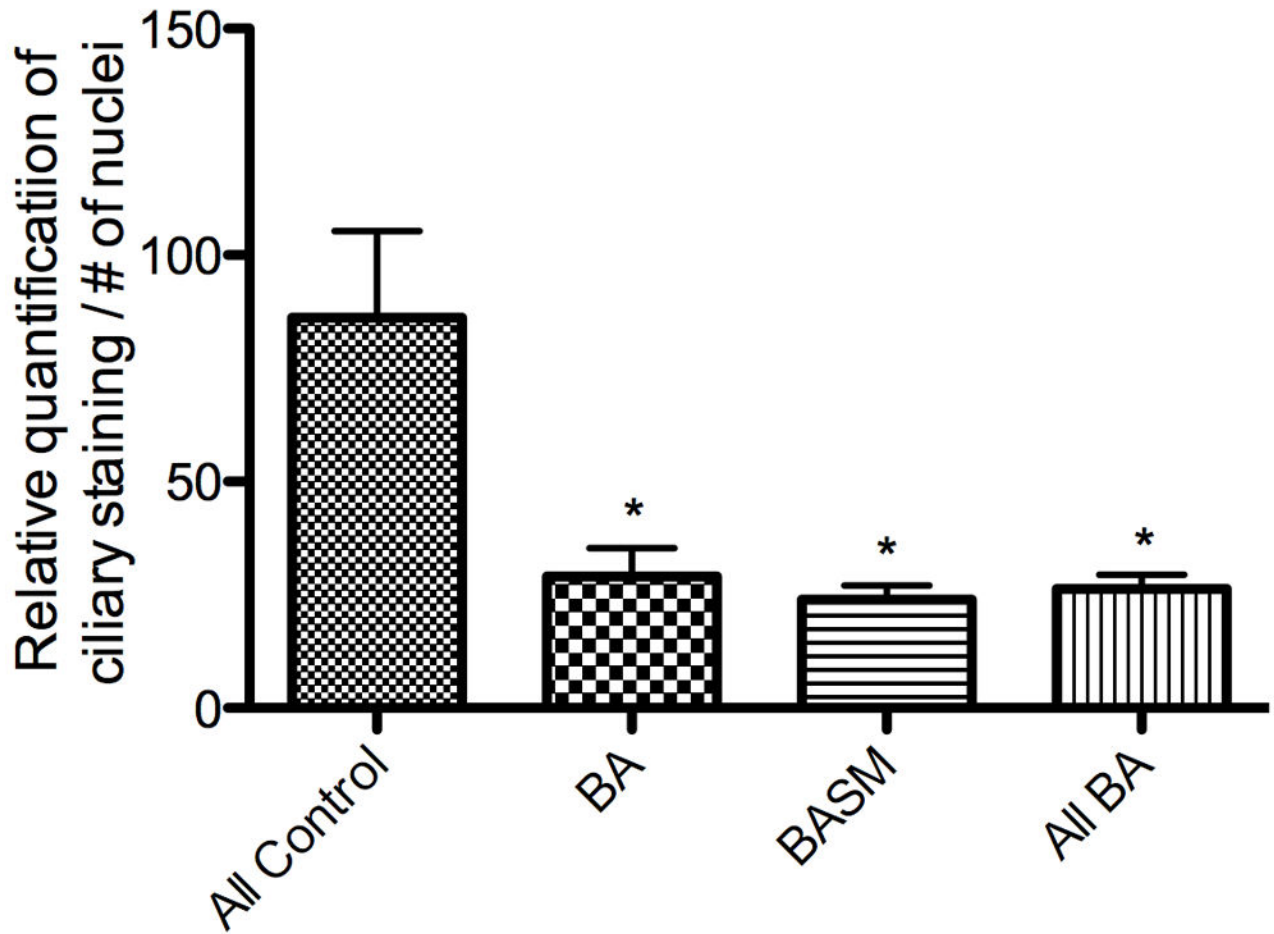


**Figure 1.** Confocal immunofluorescence microscopy of control livers. Green (Cy2) = K19 (bile ducts), red (Cy3) = acetylated  $\alpha$ -tubulin (cilia). Note the quantity, length, and orientation of the intraluminal cilia. A) Specimen C1, normal liver adjacent to hepatoblastoma; B) specimen C4, primary sclerosing cholangitis; C) specimen C5, cardiac cirrhosis; D) specimen C6, Wilson's disease. Scale bar = 20  $\mu$ m in panels A-C, 10  $\mu$ m in panel D.

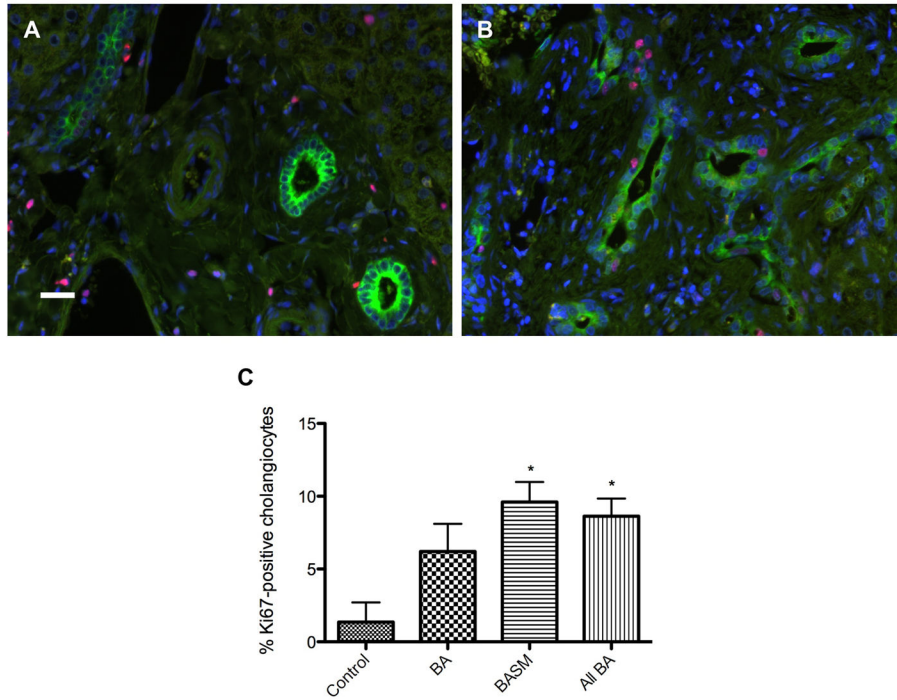




**Figure 2.** Confocal immunofluorescence microscopy of biliary atresia livers. Green (Cy2) = K19 (bile ducts), red (Cy3) = acetylated  $\alpha$ -tubulin (cilia). Note the differences in quantity, length, and orientation of the intraluminal cilia compared to Figure 1. A) Specimen B5, diagnostic wedge 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  $\mu$ m in panels A-C, 10  $\mu$ m in panel D.



**Figure 3.** Relative quantification of intraductal cholangiocyte cilia staining. For each sample, intraductal anti-acetylated  $\alpha$ -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 to 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  $\mu\text{m}$ .

**Table 1**

Sources of tissue.

Control samples			
Specimen	Pathology	Biopsy Type	Age
C1*	Normal tissue adjacent to hepatoblastoma	Resection	NA
C2*	Normal tissue adjacent to hepatoblastoma	Resection	5y
C3*	Citrullinemia	Explant	6m 22d
C4	PSC	Explant	19y
C5	Cardiac cirrhosis	Explant	9y
C6	Wilson's	Explant	17y

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

\* Histologically normal

\*\* B8a and B8b were from the same patient, taken at different times.

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