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Evidence From Human and Zebrafish That *GPC1* Is a Biliary Atresia Susceptibility Gene

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Abstract

BACKGROUND & AIMS—Biliary atresia (BA) is a progressive fibroinflammatory disorder of infants involving the extrahepatic and intrahepatic biliary tree. Its etiology is unclear but is believed to involve exposure of a genetically susceptible individual to certain environmental factors. BA occurs exclusively in the neonatal liver, so variants of genes expressed during hepatobiliary development could affect susceptibility. Genome-wide association studies previously identified a potential region of interest at 2q37. We continued these studies to narrow the region and identify BA susceptibility genes.

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Supplementary Materials

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Conflicts of interest

The authors disclose no conflicts.

METHODS—We searched for copy number variants that were increased among patients with BA (n = 61) compared with healthy individuals (controls; n = 5088). After identifying a candidate gene, we investigated expression patterns of orthologues in zebrafish liver and the effects of reducing expression, with morpholino antisense oligonucleotides, on biliary development, gene expression, and signal transduction.

RESULTS—We observed a statistically significant increase in deletions at 2q37.3 in patients with BA that resulted in deletion of one copy of *GPC1*, which encodes glypican 1, a heparan sulfate proteoglycan that regulates Hedgehog signaling and inflammation. Knockdown of *gpc1* in zebrafish led to developmental biliary defects. Exposure of the *gpc1* morphants to cyclopamine, a Hedgehog antagonist, partially rescued the *gpc1*-knockdown phenotype. Injection of zebrafish with recombinant Sonic Hedgehog led to biliary defects similar to those of the *gpc1* morphants. Liver samples from patients with BA had reduced levels of apical GPC1 in cholangiocytes compared with samples from controls.

CONCLUSIONS—Based on genetic analysis of patients with BA and zebrafish, *GPC1* appears to be a BA susceptibility gene. These findings also support a role for Hedgehog signaling in the pathogenesis of BA.

Keywords

GWA; Susceptibility Loci; Animal Model; Bile Duct Growth and Development

Biliary atresia (BA) is a progressive fibroinflammatory disorder of infants involving the extrahepatic and intrahepatic biliary tree that results in obliteration of the ducts, leading to cholestasis, fibrosis, and cirrhosis. The etiology of BA is unknown but likely involves an environmental factor acting on a genetically susceptible infant, resulting in immune dysregulation leading to progressive biliary inflammation and damage. The exclusive occurrence of BA in the neonatal liver and extrahepatic biliary tree suggests that ongoing development may be key in pathogenesis, and thus genes affecting biliary development may confer susceptibility to BA.

We are interested in determining the nature of this genetic susceptibility. Others have examined genetic factors in the etiology of BA, but these studies have been hampered by the rarity of BA and a paucity of families with a clear inheritance pattern. There are reports of familial BA,[1] but twin studies have been inconclusive.[2–4] There is clinical heterogeneity in BA, with 20% of patients showing a distinct disorder with laterality defects,[5] whereas the majority do not exhibit other anomalies.

A recent genetic study of patients with BA showed association with a region on chromosome 10,[6] but most attempts to uncover a genetic cause for BA have focused on the subset of patients with laterality defects. Examination of laterality genes in patients with BA has found no mutations to date, however.[7] Thus, previous studies on genetics in BA have not identified specific genes, but the existing evidence suggests that genetic susceptibility is plausible.

Multiple investigators have used genome-wide association (GWA) studies of either single nucleotide polymorphisms (SNPs) or copy number variants (CNVs) to identify susceptibility loci in complex disorders, including diabetes,[8] asthma,[9] and autism,[10] among others. Such studies typically identify polymorphisms associated with specific disorders, but demonstration of a functional role in pathogenesis requires biological analysis, usually in an animal model. We chose to follow up GWA studies on patients with BA with functional analysis in zebrafish.

Zebrafish are a powerful animal model well suited to this application, because rapid and ex utero development of large numbers of embryos and larvae facilitates use as a screening tool for multiple genes of potential importance. There is considerable overall conservation of developmental processes and anatomic function between zebrafish and mammals, and this conservation extends to hepatobiliary development. The anatomy of the extrahepatic biliary tree is well conserved with mammals and includes a gallbladder. By 5 days postfertilization (dpf), the zebrafish liver has distinct hepatocytes and cholangiocytes, with an interconnecting ductular network.[11] Conservation of hepatobiliary development at the molecular regulatory level is also evident,[12–14] and several biliary disease models have been established in zebrafish, including Alagille syndrome,[14] arthrogryposis–renal dysfunction–cholestasis syndrome,[15] intrahepatic BA,[16,17] and choledochal cysts.[18]

To identify genes potentially important in BA, we have performed GWA studies using SNPs and CNVs. Early findings in these studies were reported previously, in which 35 patients with BA were compared with more than 2000 controls to identify a potential region of interest at 2q37.3.[19] Here we report expansion of these studies that narrow the region to include only one gene, *GPC1. GPC1* encodes glypican 1, one of 6 members of the glypican family of heparan sulfate proteoglycans that are distinct from other proteoglycans in that they attach to the cell membrane by a glycosyl-phosphatidylinositol linkage.[20] Glypicans mediate inflammation and modulate intercellular signaling via Hedgehog, Wnt, and fibroblast growth factor.[21–23] Glypicans appear to be important in mediating duct growth and branching in developing mouse kidney[21] and *Drosophila* trachea[22] as well as in endothelial tubulogenesis.[24] These processes appear similar to biliary development in zebrafish and thus may share molecular regulatory features with mammalian biliary development.

In this study, we used zebrafish to examine the role of *gpc1* in biliary development and established mechanistic connections between our zebrafish studies and BA in patients, involving the importance of Hedgehog signaling. These studies thus not only identify *GPC1* as a risk gene for BA but also offer mechanistic insight into the potential pathogenic role of *GPC1* in BA.

Materials and Methods

Genetic Studies of BA

Sixty-one patients with BA at The Children's Hospital of Philadelphia (CHOP) were enrolled in the study under a protocol approved by the institutional review board. DNA was prepared from peripheral blood using standard extraction procedures.[19] The patients were genotyped on the Illumina Infinium II HumanHap 550 BeadChip SNP array (San Diego, CA) through the Center for Applied Genomics at CHOP. The Center for Applied Genomics also supplied genotypes of 5088 healthy controls run on the same platform. Both cohorts underwent standard quality control metrics.[25] The CNVs were called using PennCNV with GC wave correction. This algorithm predicts the copy number (CN) of the genomic regions based on the probe intensity values and B allele frequencies (eg, CN = 0, CN = 1, CN = 3, CN = 4). Because the pathogenicity of deletions and duplications is inherently different, they were evaluated separately. The CNVs called from the algorithm were summarized as CNV regions, where every region is analyzed on a 2-SNP resolution. The difference in copy number between cases and controls was assessed using a 2-tailed Fisher exact test as previously described.[26]

Zebrafish Lines

All zebrafish experiments were performed on TL zebrafish raised in the CHOP animal facility in accordance with protocols approved by the CHOP Institutional Animal Care and Use Committee.

In Situ Hybridization Studies

In situ hybridizations were performed as described previously[27] except that 0.25% acetic anhydride was added after proteinase K fixation to reduce background.[28] Polymerase chain reaction (PCR) primers used to generate riboprobes to the zebrafish *gpc* genes are shown in Supplementary Table 1. Sequences for all genes were obtained from the Sanger Centre, Zv9 (www.sanger.ac.uk).

Morpholino Knockdown Studies and Cytokeratin Immunostaining

Morpholinos to *gpc1* were obtained from Gene Tools (Philomath, OR) and are depicted in Supplementary Table 1. Morpholinos (1.5 ng) were injected at the one-cell stage, similar to previous studies, and titrated to effect. Quantitative PCR documentation *of gpc1* knockdown is shown in Supplementary Figure 1. Larvae were collected and fixed at 5 dpf.

For the Hedgehog inhibition studies, treatment with cyclopamine (20 μmol/L) was initiated at 2 dpf and larvae were maintained in cyclopamine-containing E3 until killed at 5 dpf. We also examined larvae treated in 5 μmol/L and 100 μmol/L cyclopamine, as shown in Supplementary Figure 2.

For the Sonic Hedgehog (SHH) injection studies, recombinant human SHH (CYT-676; ProSpec, New Brunswick, NJ) was diluted to 100 μg/mL and injected into the yolk of 2 dpf larvae, similar to our recent studies using interferon gamma.[29] Larvae were collected and fixed at 5 dpf.

Whole-mount cytokeratin staining was performed as previously described after fixation in methanol/dimethyl sulfoxide.[13] Duct quantification was performed identically to previous studies.[25]

Quantitative PCR Studies

Samples for quantitative real-time PCR were obtained at 5 dpf. Livers from 5 dpf larvae were isolated and pooled in groups of 10 per condition in RNAlater (Qiagen, Gaithersburg, MD). RNA was isolated and reverse transcribed per standard protocols, and quantitative PCR was performed in accordance with standard protocols using StepOne Plus (Applied Biosystems, Life Technologies, Grand Island, NY). Primers for *gli2a*, *ptch1*, *foxl1*, *znf697*, and *ccnd1* are noted in Supplementary Table 1. Normalization was performed using *hprt*. Graphs depicted are representative experiments comparing the means of quadruplicate samples per condition.

Studies on Liver Samples From Patients

Unstained liver biopsy specimens were obtained from established cases, in accordance with an approved CHOP institutional review board protocol. Immunostaining was performed by standard techniques using primary antibody against GPC1 (sc-14645; Santa Cruz Biotechnology, Santa Cruz, CA).

Results

GWA Studies of Patients With BA

To identify genes potentially important in BA, we performed a GWA study using CNVs. Previously, we investigated potentially causative large genomic alterations in 35 patients compared with more than 2000 controls and uncovered overlapping deletions in 2q37.3 in the patients with BA.[19] In the current study, we extended this work to examine association with CNVs in 61 patients and 5088 healthy controls. This analysis uncovered a CNV region on chromosome 2 overlapping the previously identified region as well as additional regions (Supplementary Table 2). The region at 2q27.3 was heterozygously deleted in 6 patients (9.84%) and 4 controls (0.08%) ($P = 4.4 \times 10^{-10}$). The associated CNV region contained only one gene, *GPC1* (Supplementary Figure 3), suggesting that *GPC1* could be a BA susceptibility gene.

Expression of Glypican Family Members in Developing Zebrafish

We elected to screen promising BA candidate genes in zebrafish. Genes with a functional role in biliary development and/or inflammation in zebrafish would be plausible candidates for a similar role in the developing human liver and would thus be attractive as potential BA susceptibility genes.

Both zebrafish and humans have 6 *GPC* genes. We examined tissue-specific expression of all 6, because zebrafish *gpc1* may not be the functional orthologue of *GPC1*. We observed liver expression of *gpc1, 3*, and *6* at *3* dpf (Figure 1), which corresponds to the initiation of active biliary growth as evidenced by rapid proliferation of cholangiocytes and formation of nascent ducts.[12,14] Genomic analysis showed synteny of these genes with their human counterparts, as did in silico analysis of amino acid sequence (not shown). Thus, we focused on *gpc1*, because this gene demonstrated liver-specific expression and was the likely orthologue of human *GPC1*.

Morpholino-Mediated Knockdown of *gpc1* Leads to Developmental Biliary Defects

To determine the importance of *gpc1* in biliary development of zebrafish, we designed morpholino antisense oligonucleotides (MOs) directed against *gpc1*. Similar to previous studies, we designed 2 nonoverlapping MOs targeting *gpc1*: an MO targeting the translation start site and an MO targeting the splice acceptor site of exon 4 (see Supplementary Table 1 for sequences). Injection of *gpc1* MO showed no gross defects in the appearance at 5 dpf compared with larvae injected with control MO (not shown). We confirmed knockdown of *gpc1* using the splice-blocker MO and showed specificity of the MO by rescuing the phenotype with coinjection of *gpc1* messenger RNA and the MO (Supplementary Figure 1).

Control and MO-injected larvae were fed the fluorescent lipid reporter PED-6 for examination of biliary function. PED-6 is swallowed, absorbed by the intestine, processed through the liver, and excreted into bile, concentrating in the gallbladder. Defects in intrahepatic biliary anatomy can lead to decreased gallbladder uptake of PED-6. Gallbladders of larvae injected with *gpc1* MO were significantly less intense than control larvae (Figure 2). Examination of intrahepatic biliary anatomy by cytokeratin immunostaining showed fewer and less complex-appearing ducts in *gpc1* morphants (Figure 2 and Table 1, *top 2 rows*), similar to previous studies.[12,15–17,27]

We also examined extrahepatic biliary anatomy in 5 dpf *gpc1* morphants using cytokeratin immunostaining. The gallbladders of *gpc1* morphants were smaller, with fewer cells (61.3 ± 11.6 for control, 34.3 ± 6.0 for *gpc1* morphants, n = 4 each, $P \leq .05$), than those of control larvae (Figure 2). The biliary defects noted in *the gpc1* morphants show that inhibition of

gpc1 leads to both extrahepatic and intrahepatic biliary anomalies, as in BA. The extrahepatic and intrahepatic defects showed equal sensitivity to MO-mediated knockdown, because both the dose shown in Figure 2 (1.5 ng) and a lower dose (0.75 ng) affected both phenotypes equally (not shown). Our results support a potential role of *GPC1* in BA.

Gene Expression Changes in *gpc1* Morphants

Recently, others have shown that Hedgehog activity is increased in patients with BA[30] and, as mentioned previously, glypicans modulate signaling via Hedgehog. Thus, we examined the expression of Hedgehog target genes in *gpc1* morphants. Expression of the target genes *gli2a,ptch1*, *foxl1*, *znf697*, and *ccnd1*[31] was increased at 5 dpf in livers from *gpc1* morphants (Figure 3). In addition, several genes associated with fibrogenesis and epithelial-mesenchymal transition, a process linked to increased Hedgehog activity, were up-regulated in livers from *gpc1* morphants (Supplementary Figure 4). This suggested that Hedgehog activity was increased in the livers *of gpc1* morphants, consistent with a model in which loss of *gpc1* leads to activation of Hedgehog signaling.

Inhibition of Hedgehog Signaling Partially Reverses Biliary Defects in *gpc1* Morphants

To determine the importance of increased Hedgehog activity in mediating the biliary defects in the *gpc1* morphants, we treated *gpc1* morphants with the Hedgehog inhibitor cyclopamine. This resulted in partial rescue of the intrahepatic biliary defects caused by *gpc1* knockdown (Figure 4 and Table 1, *bottom row*), suggesting that increased Hedgehog activity is at least partially responsible for the biliary defects seen in *gpc1* morphants. There was no effect of cyclopamine on control larvae (data not shown), but injection of SHH protein into developing larvae led to abnormalities in bile duct morphology (Figure 4), consistent with overactive Hedgehog signaling, leading to developmental biliary defects. The rescue of *gpc1* knockdown by Hedgehog inhibition is consistent with a model in which glypicans modulate Hedgehog activity by acting as a "sink," decreasing the availability of ligand; thus, absence of glypican results in increased Hedgehog signaling.

Patients With Cholestatic Disease Have *GPC1* Abnormalities

Our zebrafish studies support a role for *GPC1* in mediating biliary defects in patients. To examine the potential role of GPC1 in patients with BA, we performed immunostaining on liver samples. In control patients, GPC1 staining was localized to the apical surface of cholangiocytes. GPC1 staining was also localized to the apical surface of cholangiocytes from patients with cystic fibrosis liver disease, autosomal recessive polycystic kidney disease, and primary sclerosing cholangitis (PSC). In contrast, in liver samples from patients with infantile cholestasis, including patients with BA and patients with total parenteral nutrition (TPN)-associated cholestasis, no apical GPC1 staining was visible and the amount of GPC1 appeared to be decreased globally (Figure 5). Of note, the patients with BA shown in Figure 5 were not among those with deletions in the *GPC1* region. These results show localization and/or quantification defects of GPC1 in patients with infantile cholestasis, supporting the importance of GPC1 in BA that could be mediated genetically.

Discussion

In this study, we showed that the proteoglycan gene *GPC1* is a potential risk gene for BA, with evidence from genetic studies of patients with BA and from knockdown of *gpc1* in zebrafish. We showed that the biliary defects elicited by *gpc1* knockdown are reversed by inhibition of Hedgehog signaling and that patients with BA have abnormalities in GPC1 immunostaining. These results support the potential importance of *GPC1* in BA and also show the utility of screening candidate genes uncovered in genetic association studies using zebrafish.

BA and Genetic Susceptibility

This is the first study to identify a potential BA risk gene in patients and to show functional defects in the biliary system in model organism studies. Previously, Garcia-Barcelo et al identified association of BA with a region on chromosome 10q24.2.[6] This region lies between *XPN-PEP1*, encoding X-prolyl aminopeptidase P, and *ADD3*, which encodes adducin 3. X-prolyl aminopeptidase P catalyzes the breakdown of bradykinin and substance P, whereas adducin 3 is a cytoskeletal protein involved in cell motility and cell-cell contact. Both genes are expressed in biliary cells, and both proteins have functions potentially important in BA.

Other studies have adopted a more direct approach, examining the role of specific genes in the pathogenesis of BA based on overlap of clinical features with other disorders or genes and pathways believed to be important in the pathogenesis of BA. Such candidates include *JAG1*, the primary gene involved in Alagille syndrome, as well as laterality genes and inflammatory pathway genes. Kohsaka et al found *JAG1* mutations in 9 of 102 patients with BA, and these patients appeared to have more severe disease.[32] Because of the association between BA and laterality defects,[5] investigators have examined patients with BA for mutations in genes involved in left/right sidedness. No mutations in *INV* were identified in 7 patients with BA,[7] whereas *CFC1* mutations were found in 5 of 10 patients with BA and laterality defects,[33] although the significance of these mutations is not clear. Investigators have also examined genes important in inflammation. Polymorphisms in the gene for macrophage migration inhibitory factor (*MIF*) have been described in patients with BA.[34] Lee et al identified polymorphisms in the vascular endothelial growth factor (*VEGF*) gene associated with BA,[35] and this group also reported no polymorphisms in *IFNG*,[36] *IL4*,[37] or *IL18*,[38] in patients with BA. Although examination of specific genes may lead to identification of some susceptibility genes, these studies are hampered by a relatively small sample size and a necessary gene selection bias that large-scale GWA studies attempt to circumvent. Additionally, because of the likely genetic heterogeneity in BA, different genes may confer susceptibility in different patients.

Specific genetic disorders have been reported to be associated with BA or a BA-like phenotype. We have reported BA in a patient with Mowat–Wilson syndrome[16] and in a patient with trifunctional protein deficiency.[39] Although most patients with these disorders do not have liver disease, the simultaneous occurrence of BA and a rare genetic disorder suggests a possible role for the causative gene in mediating BA.

Thus, our current study adds to a growing list of potential BA susceptibility genes (Table 2).[16,18,27,40–53] In addition to the genes discussed previously, Table 2 lists genes that cause other disorders with biliary defects. Patients with North American Indian childhood cirrhosis have liver disease similar to that in patients with BA; this disorder is caused by homozygous mutation of *CIRH1A*.[54] In addition, there are other genetic cholestatic disorders, such as ARC syndrome[45] and Aagenaes syndrome.[55] There are multiple conditions, including Joubert syndrome, Meckel syndrome, and Bardet–Biedl syndrome, involving genes encoding proteins important in primary cilia.[40] In these disorders, biliary morphogenesis defects are associated with other findings, including laterality defects. Although the ciliopathies have not been linked to BA, genes in these disorders would be excellent BA susceptibility gene candidates. More extensive GWA studies may show that the genes in Table 2 are associated with BA and will also likely uncover unexpected genes. Continued screening of such genes using zebrafish, as reported here, will strengthen the connection to BA.

Our studies have focused on a genetic association with BA, but we cannot rule out that genetic abnormalities in *GPC1* are a risk factor for infantile cholestasis or for cholestasis in

general. Our immunostaining studies showing GPC1 abnormalities in TPN cholestasis support the former. With respect to the latter, others have examined genetic influences on other cholestatic diseases such as PSC and primary biliary cirrhosis. Studies examining PSC have not shown association with SNP variants in *GPC1*[56–59] and similar studies on primary biliary cirrhosis are likewise negative,[60–62] but there may be a modest association between PSC and decreased *GPC1* CN (E. Ellinghaus and T. Karlsen, personal communication, December 2012). Interestingly, polymorphisms in *GPC6* are associated with PSC, and lentiviral silencing of *GPC6* in cholangiocytes leads to activation of proinflammatory genes.[59] These latter studies, along with our studies, support the importance of glypican abnormalities in cholestasis in general and suggest that there may be genetic defects in *GPC* genes in multiple forms of cholestasis.

Glypicans and Morphogenesis Defects

We showed that knockdown of *gpc1* leads to biliary developmental defects in zebrafish and that these defects appear at least partly due to Hedgehog signaling abnormalities. In patient samples, we observed a decrease in GPC1 at the apical surface of cholangiocytes in patients with BA. This decrease could occur via a genetic lack of *GPC1* or secondary to other changes in the cholangiocyte in BA and similar disorders, such as loss of cell polarity and/or epithelial-mesenchymal transition.

Glypicans are proteoglycans anchored in the apical membrane and have been shown to modulate both Hedgehog and fibroblast growth factor signaling. There is evidence for glypicans to either augment or attenuate this signaling. Based on our data, GPC1 could act as an inhibitor for Hedgehog ligands, similar to reports previously for GPC3.[63] Reduced GPC1 would then increase Hedgehog signaling.

Such a model would require the presence of Hedgehog ligand in the bile, because our evidence in patients shows GPC1 expression in the lumen. Although there is as yet no evidence for Hedgehog ligands in bile, recent studies have shown the presence of fibroblast growth factor 19 in bile,[64] suggesting that other signaling proteins that interact with glypicans exist in bile. Bile salts are important signaling molecules found in bile, but the presence of protein signaling molecules in bile suggests a previously unappreciated medium for signaling potentially disrupted in cholestatic diseases such as BA.

With respect to the mechanism by which *gpc1* and Hedgehog signaling disrupt biliary development in zebrafish, others have shown activation of the Hedgehog target gene *Foxl1* in rodent models of cholestasis,[65] and we showed increased *foxl1* expression in *gpc1* morphants. The *Foxl1* gene is expressed in hepatocyte and cholangiocyte bipotential precursors,[66] supporting a potential role for this genetic pathway in mediating biliary defects, because disruption of this pathway may negatively affect differentiation. Other potential downstream targets of Hedgehog, including transcription factors such as *znf697*, may also be important. Further examination of these targets may not only reveal genes important in biliary development but also may uncover additional BA susceptibility genes.

Zebrafish as a Genetic Screening Tool

Zebrafish have repeatedly shown utility as a high-throughput screening tool in forward genetic screens, small molecule screens, and other types of screens. Here we reported use of zebrafish in conjunction with GWA studies as a method to screen genes close to SNPs associated with a particular disease. To be truly useful as a screening tool for GWAS, potentially affected zebrafish require a facile functional assay, such as PED-6 screening and cytokeratin immunostaining. Given the multiple methods available to analyze larval zebrafish, numerous clinical conditions may be amenable to similar analysis.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations used in this paper

BA	biliary atresia
CHOP	The Children's Hospital of Philadelphia
CN	copy number
CNV	copy number variant
DAPI	4′,6-diamidino-2-phenylindole
dpf	days postfertilization
GWA	genome-wide association
MO	morpholino antisense oligonucleotide
PCR	polymerase chain reaction
PSC	primary sclerosing cholangitis
SHH	Sonic Hedgehog
SNP	single nucleotide polymorphism
TPN	total parenteral nutrition

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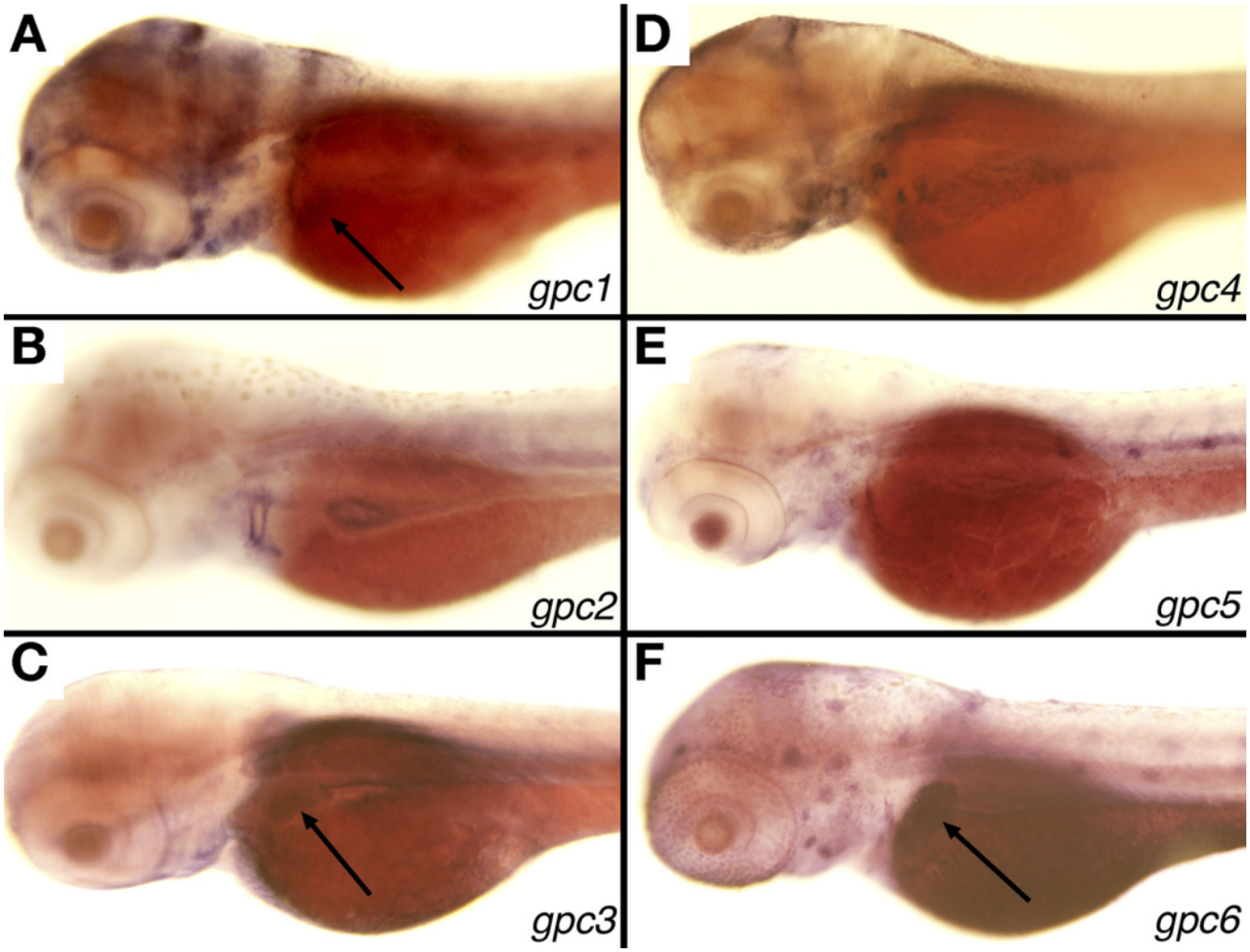

Figure 1.
Expression of *gpc* genes in larval 3 dpf zebrafish. In situ hybridization studies of 3 dpf zebrafish showing liver expression (*arrows*) of (*A*) *gpc1*, (*C*) *gpc3*, and (*F*) *gpc6*, but not in *gpc2* (*B*), *gpc4* (*D*), or *gpc5* (*E*). There is also faint intestinal expression of all 6 genes.

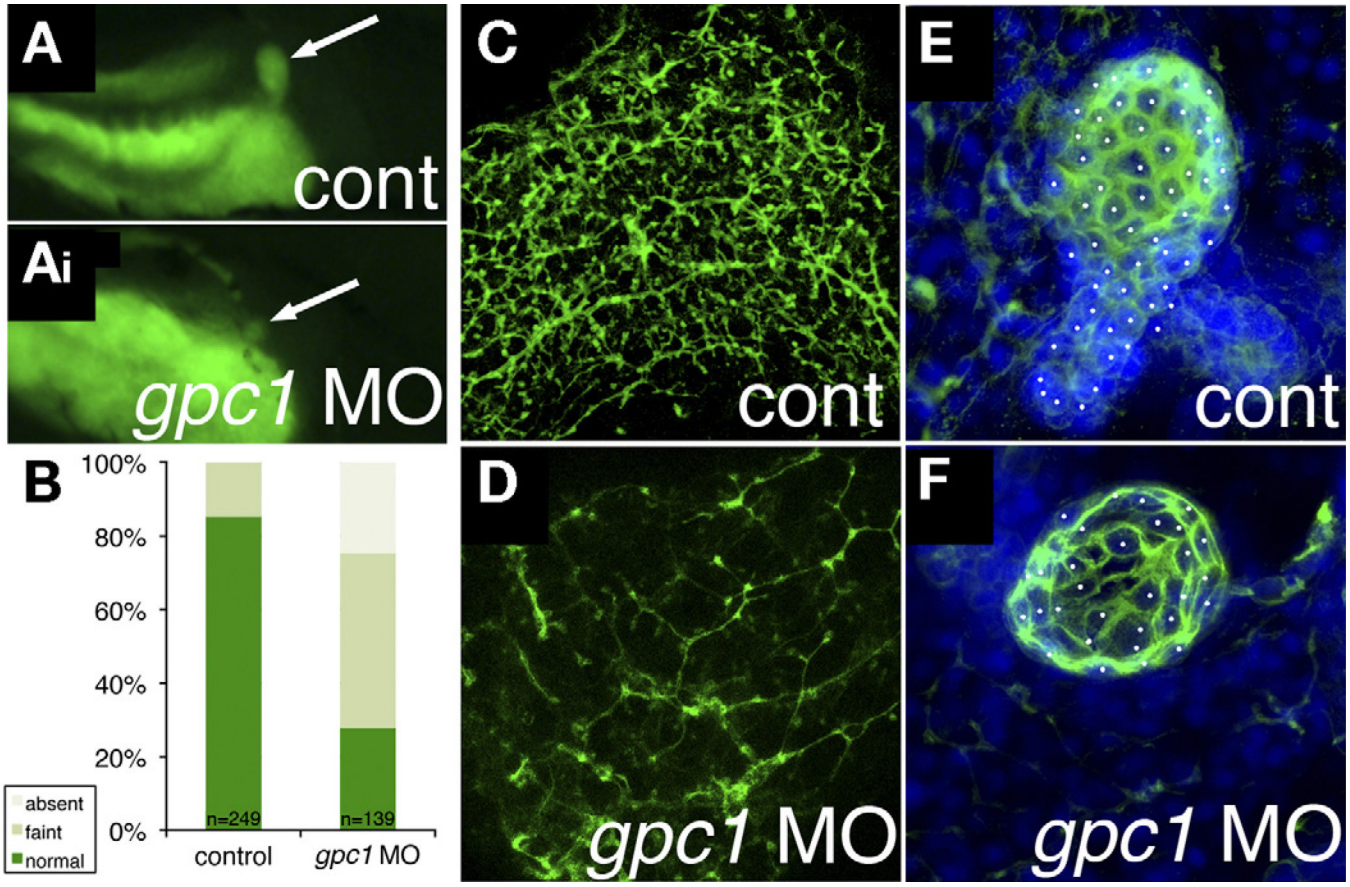

Figure 2.

Knockdown of *gpc1* in zebrafish leads to developmental biliary defects. (*A, Ai*) Right lateral views of 5 dpf zebrafish larvae after ingestion of PED-6, showing a more intense and larger gallbladder in (*A*) control than in (*Ai*) the *gpc1* morphant. (*B*) Quantification of PED-6 uptake in control and *gpc1* morphant larvae, showing a highly significant ($P < .0001$, χ^2) decrease in gallbladder intensity in the *gpc1* morphants. (*C* and *D*) Confocal projections of whole-mount cytokeratin immunostaining of livers from 5 dpf (*C*) control and (*D*) *gpc1* morphant, showing decreased number and complexity of the ducts in *panel D*. (*E* and *F*) Confocal projections of gallbladders stained for cytokeratin and counterstained with 4',6-diamidino-2-phenylindole (DAPI), with cells noted by *white dots*. Note that there are more cells in *panel E*.

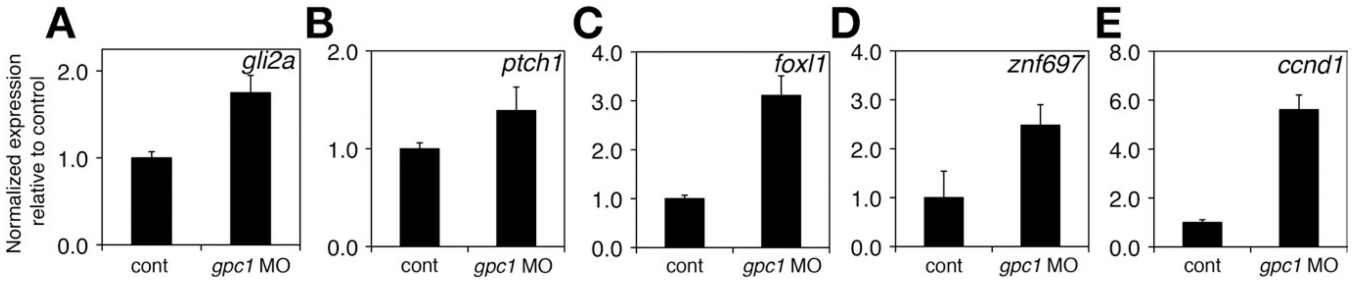

Figure 3.

Expression of downstream genes in *gpc1* morphants. (*A*–*E*) Quantitative PCR studies of Hedgehog target genes in isolated livers from 5 dpf larvae show increased expression of (*A*) *gli2a*, (*B*) *ptch1*, (*C*) f*oxl1*, (*D*) *znf697*, and (*E*) *ccnd1*. $P < .05$ for all.

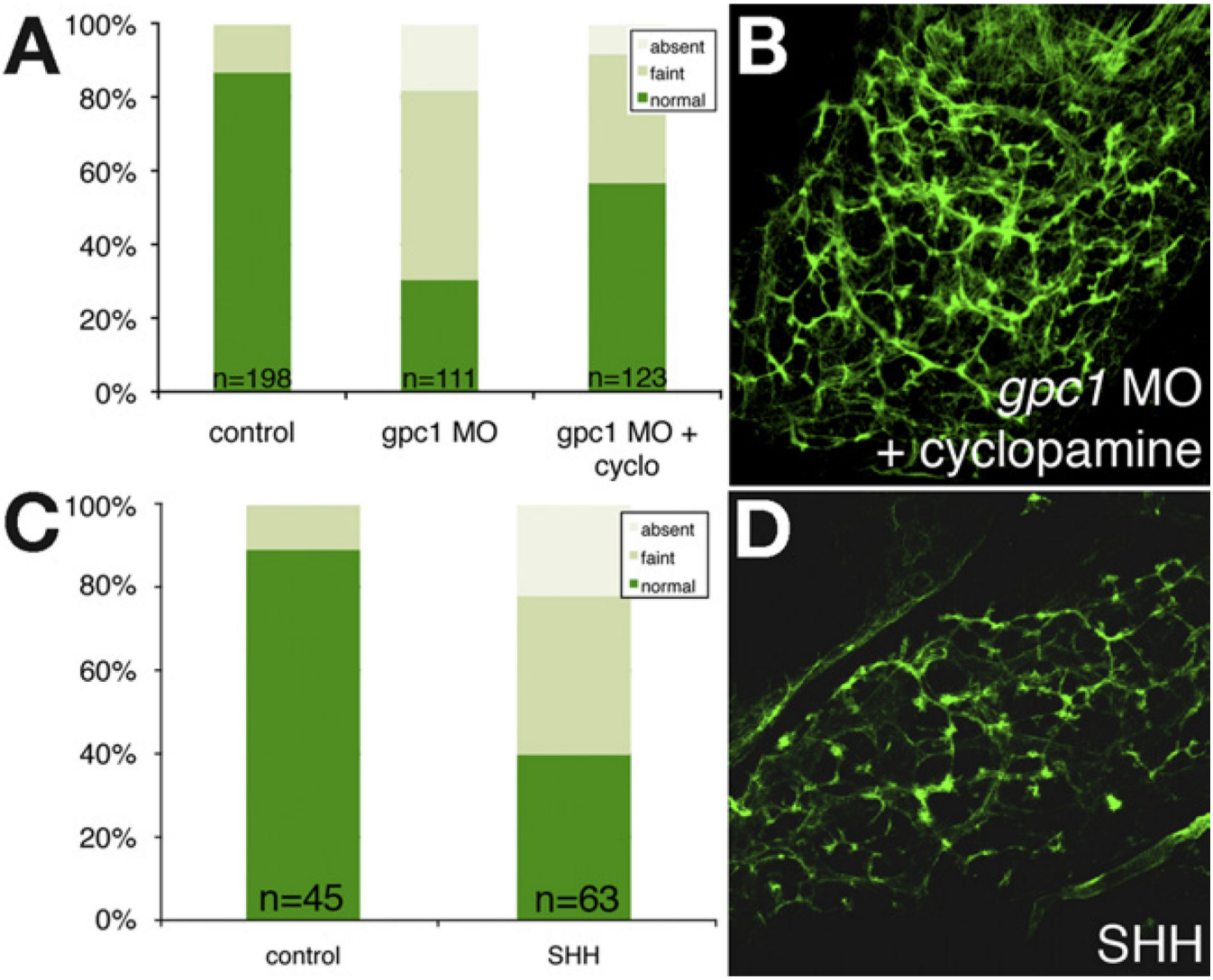

Figure 4.

Increased Hedgehog activity is associated with biliary defects. (*A*) Quantification of PED-6 uptake in control, *gpc1* morphant larvae, and *gpc1* morphant larvae treated with cyclopamine. There is a significant reduction in gallbladder intensity in the *gpc1* morphants ($P < .0001$, χ^2) that is partially rescued by treatment with cyclopamine ($P < .0001$). (*B*) Confocal projection of whole-mount cytokeratin immunostaining of liver from a 5-dpf *gpc1* morphant larva treated with cyclopamine. Compare with Figure 2. (*C*) Similar quantification of PED-6 uptake in control and SHH-injected larvae, showing a significant ($P < .0001$) reduction in PED-6 gallbladder uptake in SHH-injected larvae. (*D*) Confocal projection of whole-mount cytokeratin immunostaining of liver from a5-dpf SHH-injected larva, showing decrease in number and complexity of intrahepatic ducts.

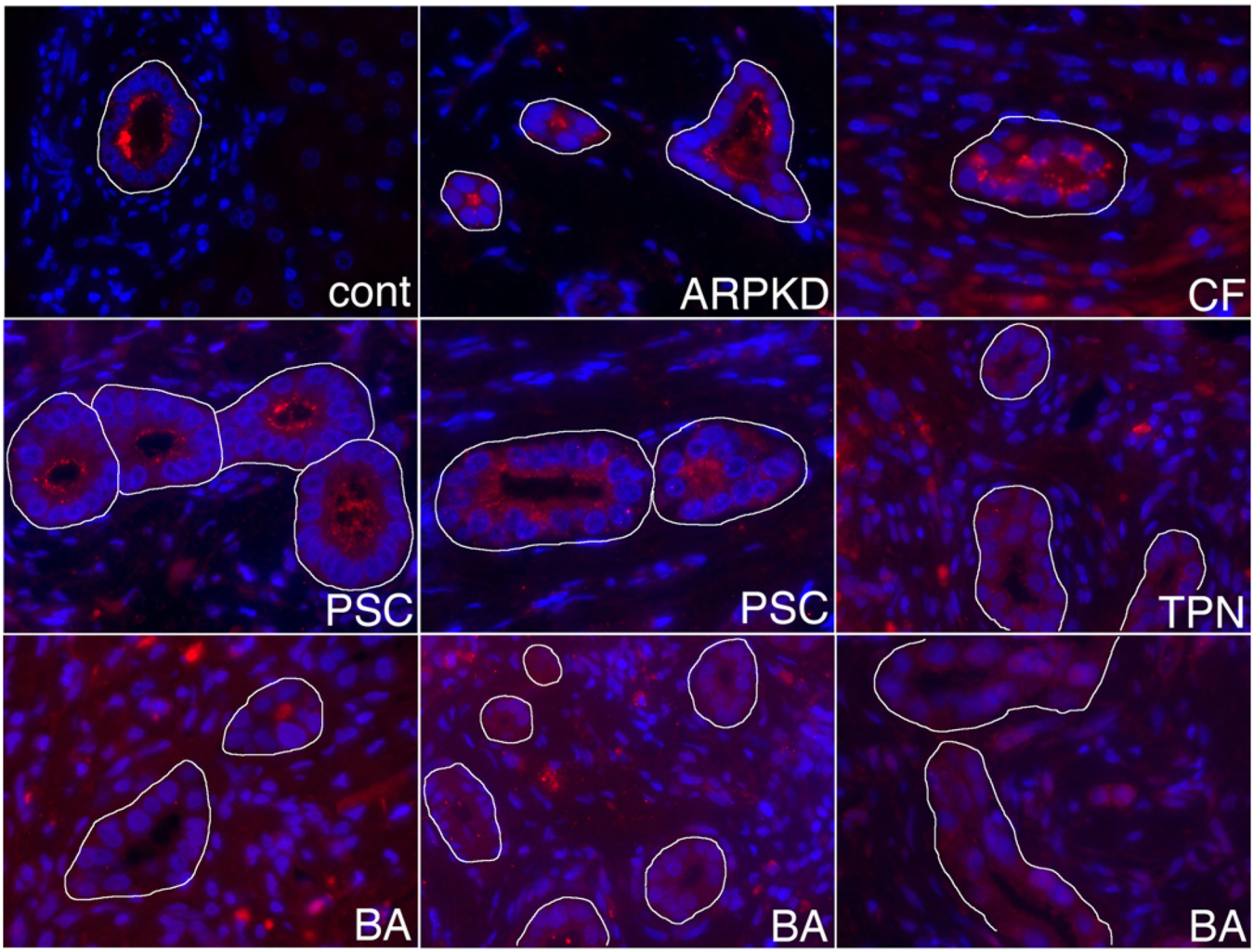

Figure 5.
GPC1 abnormalities in patients with cholestatic liver disease. Shown are GPC1 immunostainings from a control patient (cont), a patient with autosomal recessive polycystic kidney disease/congenital hepatic fibrosis (ARPKD), a patient with cystic fibrosis liver disease (CF), 2 patients with PSC, a patient with TPN-induced cholestasis, and 3 patients with BA. GPC1 staining is *red*, and samples were counterstained with DAPI. Bile ducts are outlined in *white*. Note that GPC1 staining is more diffusely localized and fainter in the TPN and BA samples.

Table 1

Quantification of Bile Ducts in *gpc1* Knockdown

	No. of total ducts	No. of interconnecting ducts	No. of terminal ductules	Duct length (*arbitrary units*)
Control	25.5 ± 3.0	6.7 ± 3.3	54.5 ± 15.7	1.45 ± 0.16
gpc1 MO	19.0 ± 4.9[a]	1.5 ± 1.4[a]	14.7 ± 9.5[b]	1.20 ± 0.39
gpc1 MO + cyclopamine	30.8 ± 10.9[a]	5.3 ± 3.8[a]	31.2 ± 12.2[a]	1.18 ± 0.12

NOTE. Duct attributes from control and *gpc1* MO-injected larvae. Shown are the number of total ducts, interconnecting ducts, terminal ductules, and duct length, measured in arbitrary units.

[a] $P \leq .05$,

[b] $P \leq .005$,

gpc1 MO relative to control. Also shown is quantification of duct features from *gpc1* MO-injected larvae treated with cyclopamine. [a] $P \leq .05$, relative to *gpc1* MO alone.

Table 2

Genes Associated With BA, Other Cholestatic Disorders, and Biliary Development

Gene (reference)	Function
Associated with BA	
GPC1 (this report)	Proteoglycan
XPNPEP1[6]	Aminopeptidase
ADD3[6]	Actin-binding
JAG1[32]	Cell fate determinant (Jagged/Notch)
CFC1[33]	Left/right determinant
MIF[34]	Cytokine
VEGF[35]	Growth factor
ZEB2[16]	Transcription factor
HADHA[39]	Metabolic enzyme
Genetic causes of cholestasis/bile duct morphogenetic defects	
CIRH1A[54]	Unknown
VIPAR[44] *VPS33B*[45]	Intracellular trafficking
PKHD1, BBS, MKS, NPHP, others[40,41]	Cilia structure/function
CFTR[47]	Chloride channel
SERPINA1[48]	α1-antitrypsin
ATP8B1, ABCB11, ABCB4[49]	Bile component transport
AKR1D1, CYP7B1, HSD3B7, others[50]	Bile acid synthesis
Animal models of cholestasis/bile duct morphogenetic defects	
ONECUT1[42]	Onecut transcription factor
TCF2[43]	Homeodomain transcription factor
FOXF1[46]	Forkhead transcription factor
VPS genes[15,18,51]	Intracellular trafficking
ATP6 genes[52]	Intracompartmental pH
ENO1[53]	Enolase A
INV[7]	Left/right determinant
DNMT1[17]	DNA methylation
PRICKLE1, VANGL2[27]	Planar cell polarity

NOTE. The genes are divided into 3 categories. The first group shows genes identified by linkage studies, direct examination of the gene in patients with BA, or reports of patients with a known mutation also with BA. The second group has genes responsible for diseases with a hepatic or biliary phenotype, and the third group has genes that lead to hepatobiliary defects in animal models.