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Fishing for Biliary Atresia Susceptibility Genes

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Biliary atresia (BA) is a devastating neonatal cholestatic disease in which fibroinflammatory obstruction of the extrahepatic biliary tree rapidly causes biliary fibrosis. If uncorrected, BA results in death within the first two years of life. If BA is diagnosed within the first 4 months of age, Kasai portoenterostomy (KPE) is typically performed to restore biliary drainage, which is successful in less than half of the children. In a contemporaneous North American cohort of 244 patients with BA who underwent KPE and were prospectively enrolled into the multi-center Childhood Liver Disease Research and Education Network (ChiLDREN), only 46.7% survived with their native liver beyond 2 years of age¹. The reason for the failure of KPE in many patients is unclear, but it is believed to be due in part to progressive intrahepatic bile duct injury and impaired repair of bile duct epithelial cells. Eventually, 70–80% of BA patients require liver transplantation to prolong survival. Attempts to develop adjuvant medical therapies to improve the surgical outcome are hampered by limited understanding of disease mechanisms controlling initiation and progression of BA.

BA is likely a multi-factorial disorder in which an environmental trigger, i.e. virus infections² or toxins³, initiate an aberrant hepatic immune response in a genetically susceptible host (Figure 1). Application of genome wide association studies (GWAS) to identify pathways relevant for disease-specific cholangiocyte injury in BA may be of benefit, as in other immune mediated liver diseases⁴⁵. The concept that genetic susceptibility factors predispose to BA is supported by several observations, in particular the differences in incidence depending on ethnic and racial background, which reaches up to 1/5000 Asian infants but only 1/18,000 Caucasian infants. In contrast, discordant twin studies and the striking heterogeneity in clinical presentation and response to surgical therapy argue against a monoallelic condition and indicate the existence of risk alleles for different subtypes of BA. Nonetheless, genes coordinating hepatobiliary development appear to be candidates, especially since the disease is uniquely restricted to embryonic (Biliary Atresia Splenic Malformation syndrome) and early postnatal periods. In a recent GWAS of a North American cohort of 35 subjects with BA, a heterozygous deletion spanning 30 genes of 2q37.3 was detected in two patients⁶. In this issue of *GASTROENTEROLOGY*, Ciu et al. extended this GWAS by examining copy number variations in additional 31 subjects with BA and in 5000 controls, detecting heterozygous deletions spanning the same locus in four

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more subjects and identifying GPC1 encoding glypican 1, as a potential susceptibility factor for BA.

Glypicans (GPCs) are cell surface heparan sulfate proteoglycans linked to the plasma membrane by a glycosyl-phosphatidyl inositol anchor⁷. Zebrafish and humans have six GPCs that are mainly targeted to the apical surface of epithelia⁸. GPCs have the ability to modulate the range and activity of many developmental signals including Hedgehog (Hh), Wingless (Wnt), fibroblast growth factor (FGF) and bone morphogenetic protein (BMP)⁷. Each of these pathways has been implicated to play at least one major role in hepatogenesis⁹. BMP signals from the septum transversum mesenchyme (STM) and FGF signals from the cardiac mesoderm induce the hepatic diverticulum from the foregut endoderm. Hepatic bipotential progenitor cells (i.e., hepatoblasts) delaminate from the endoderm and migrate into the STM. Wnt and Hh signals are known to drive expansion of hepatoblasts^{10–12}. To establish the intrahepatic biliary architecture, hepatoblasts differentiate along the cholangiocyte (i.e., biliary epithelial) lineage if in direct contact with the mesenchyme surrounding portal veins¹³. Thus, careful orchestration of signals between epithelial and mesenchymal cells is required to guide hepatogenesis.

To explore a functional relationship between the genetic changes in *GPC1* and pathogenesis of BA, Cui et al. investigated the requirement of GPC1 in biliary development taking advantage of *in vivo* zebrafish imaging and targeted morpholino antisense oligonucleotide-mediated knockdown technologies. Knockdown of *gpc1* throughout zebrafish development leads to intrahepatic biliary and gallbladder defects observed at the late larval stage five days post fertilization (dpf). During normal embryogenesis, at 5 dpf the zebrafish intrahepatic main ducts, interconnecting branches and terminal ductules are present. In *gpc1* morphants, immunostaining for cytokeratin 18 (CK18) implies that fewer cholangiocytes are contributing to a less complex architecture when compared to controls. Bile secretion is significantly reduced in *gpc1* morphants as assessed by fluorescent lipid reporter PED-6 presence in the gallbladder and quantitative analysis indicated that fewer CK18+ cells contribute to the gallbladder at 5 dpf. These results can be interpreted as a defect in initial specification and/or an inability to maintain CK18+ cells when GPC1 is deficient. These are intriguing findings that open new opportunities for future studies to understand how GPC1 regulates biliary development and its potential implication for pathogenesis of BA. For example, it is unclear whether non-CK18+ cholangiocytes are potentially present or if there are abnormalities in extrahepatic bile ducts. Better understanding of immunostaining characteristics of cholangiocytes during the development of zebrafish may help reconcile an apparent contradiction: *gpc1* morphants develop *fewer intrahepatic bile ducts*, whereas *bile ductular proliferation* is considered the hallmark of the intrahepatic lesion of BA at diagnosis in humans. Regarding infants with BA, the ductal response to obstruction is dynamic, with an initial peak of ductular proliferation at 200 days of age followed by a phase of regression leading to paucity of intrahepatic bile ducts in uncorrected BA by 400 days of life¹⁴. How these time points relate to the phase of bile duct development examined in the zebrafish model requires further investigation.

Given that increased Hh activity is observed in patients with BA¹⁵ and GPCs are known to modulate Hh activity, Cui et al. performed comparison expression studies of known Hh target genes (*gli2a*, *ptch1*, *flox11*, *znf697*, and *ccnd1*). Hh target gene expression is increased at 5 dpf in livers from *gpc1* morphants. To functionally test whether increased Hh activity is the basis of the GPC1 deficient phenotype, *gpc1* morphants were treated with the Hh inhibitor cyclopamine. Antagonism of the Hh pathway resulted in a partial rescue of PED-6 presence in the gallbladder. Additionally, activating the Hh pathway by injection of Sonic Hedgehog protein into the developing larvae phenocopies GPC1 deficiency, with significant decreased PED-6 uptake and visibly less CK18 immunostaining. These data indicate that

increased Hh is partially responsible for the biliary defects observed in *gpc1* morphants. Interestingly, the reported physical examination for one of two patients with BA and heterozygous 2q37.3 deletion including the GPC1 locus suggests Hh associated malformations such as, digit syndactyly, umbilical hernia, and a submucosal cleft palate⁶. It will be important to determine if the newly identified four patients in this study also presented with Hh-like phenotypes. Currently, there are multiple studies demonstrating a role for Hh pathway activation during various types of adult liver injury¹⁶. No mammalian studies to date have conclusively demonstrated a paracrine or autocrine role for the Hh pathway during biliary development. Therefore, careful temporal studies investigating the lineage requirement of the Hh pathway during cholangiocyte specification and biliary architecture formation are warranted. Moreover, the ability of GPC1 to intricately modulate the level and spatial accessibility of Hh, FGF, BMP, and Wnt signals to guide complex biological outcomes remains to be evaluated.

In support of GPC1 playing an autocrine role in the cholangiocyte lineage, Cui et al. show that GPC1 staining is localized to the apical surface of cholangiocytes. The apical localization is disrupted in patients with BA and in patients with total parenteral nutrition-associated cholestasis. However, the apical localization is normal in patients with cystic fibrosis liver disease, autosomal recessive polycystic kidney disease, and PSC-associated cholestasis. The ages of the subjects are not reported and it remains to be seen whether the findings are confounded by age-difference between the groups, with BA typically occurring in infants and PSC manifesting itself in adolescents. Another aspect that requires additional studies is the potential role of GPC1 regulating immune responses targeting cholangiocytes. Of note, GWAS in PSC, which shares many similarities of large duct obstruction and biliary fibrosis with BA, detected a risk allele in GPC6, linked *in vitro* to up-regulation of pro-inflammatory cytokines⁴. Furthermore, sequence alterations in GPC5 were found to confer susceptibility to immune-mediated tissue injury in nephrotic syndrome¹⁷ or multiple sclerosis¹⁸. Studies in the murine model of virus induced BA may be required to delineate a potential role of proteoglycans in controlling immune responses targeting intra- and extrahepatic bile ducts.

The study by Ciu et al. demonstrated feasibility of a powerful platform using morpholino-treated zebrafish to assign biological significance to results of genomic studies in affected patients. Given the reduced cost of next-generation sequencing, and resources of national registries like ChiLDREN collecting data and biomaterial of well-phenotyped patients in larger cohorts than ever before, GWAS and whole-exome sequencing studies in families are likely to reveal more genetic associations. We will thus experience a greater need for biological systems, like the zebrafish model, to test for biological relevance of these findings in rapid, simple, and reproducible fashion. If development and refinement of these systems can keep pace with the advancement in next-generation sequencing technology and bioinformatics, personalized therapy for heterogeneous and rare conditions like BA may finally be within reach. If so, Dr. Everett Koop's daunting verdict from 1976: "we can say with certainty that the jaundiced baby who has had no extrahepatic bile duct has been the most disappointing patient for the surgeon in the whole realm of lesions theoretically correctable by a surgical procedure"¹⁹ may finally be overcome.

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Figure 1.
Pathogenesis of Biliary Atresia from a human and animal model perspective.