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Notch signaling promotes ductular reactions in biliary atresia

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ABSTRACT

Background: Biliary atresia (BA) is a congenital, progressive, fibro-obliterative disease of the extrahepatic biliary tree and the most common cause of end-stage liver disease in children. BA is characterized by extensive intrahepatic proliferating ductular reactions that may contribute to biliary fibrosis. Lineage tracing during experimental cholestasis indicates that cells within ductular reactions derive from PROM1-expressing hepatic progenitor cells. Given the role of Notch signaling in normal biliary development, we hypothesize that activated Notch signaling promotes the formation of ductular reactions in BA.

Methods: Liver samples collected from BA infants at Kasai portoenterostomy and age-matched controls, as well as from wild-type and *Prom1* knockout mice with 3,5-diethoxycarbonyl-1,4-dihydrocollidine (DDC)-induced experimental cholestasis were analyzed histologically using immunofluorescence and by quantitative polymerase chain reaction.

Results: Increased expression of genes encoding Notch ligand *JAG1* and its receptor *NOTCH2* was observed in BA livers compared with control by quantitative polymerase chain reaction analyses. Livers of DDC-treated mice, which exhibit cytokeratin-19–positive ductular reactions typical of BA livers, demonstrated significant increases in the expression level of the gene encoding *Notch2*, as well as downstream Notch target gene *Hes1* compared with control. *Prom1* knockout mice exhibit diminished ductular reactions and decreased levels of *Jag1* and *Hes1* compared with littermate controls.

Conclusions: Human BA and cholestasis induced by DDC are associated with Notch signaling activation. Null mutation of *Prom1* is associated with decreased ductular reactions and decreased Notch signaling activation during DDC treatment. These data are consistent with Notch signaling promoting ductular reactions of *Prom1* expressing progenitor cells in BA.

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Background

Biliary atresia (BA) is a congenital, progressive, fibro-obliterative disease of the extrahepatic biliary tree and the most common cause of end-stage liver disease in children.[1] Its incidence is approximately one in 10,000-15,000 infants in the United States.[2] Without surgical management with the Kasai portoenterostomy (KPE), children die by the age of 2 y from the rapidly progressive biliary cirrhosis.[3] Even after KPE, only 15%-20% of children will reach adolescences with their native livers, and many will manifest complications of their progressive disease such as cirrhosis and portal hypertension.[2]

Although there has been extensive research into etiology, the pathogenesis of BA remains unclear.[4] Furthermore, little is known about why intrahepatic biliary fibrosis progresses in spite of adequate surgical drainage. Prominin-1 (Prom1), also known as CD133, is a penta-transmembrane glycoprotein expressed by stem and cancer cells in multiple organs, including the liver.[5-12] We previously demonstrated the expansion of cells expressing the stem/progenitor cell marker Prom1 within regions of developing periportal fibrosis in BA.[5] Lineage tracing during experimental cholestasis caused by bile duct ligation indicates that biliary cells within ductular reactions, or proliferating ductular hyperplasia, associated with fibrosis derive from *Prom1*-expressing cells.[13,14] Moreover, ductular reactions have been implicated in transforming growth factor β (TGF-β)-mediated liver fibrosis.[15,16]

Notch signaling is an evolutionarily conserved cell-to-cell signaling cascade that is critical for biliary development and repair via the induction of hepatic progenitor cell (HPC) differentiation into biliary epithelium.[17-23] The cell-to-cell interaction involves between ligands such as Jagged and delta-like ligand and the Notch receptors.[17] Mutations in the genes encoding the Notch ligand *Jagged1* and receptor *NOTCH2* cause Alagille syndrome leading to a paucity of bile ducts.[18,20] Jagged1 has been observed in biliary epithelium and adjacent small vessel endothelium; biliary-specific expression of delta-like ligand-4 (Dll4) has been observed in human liver.[24] Of the four Notch receptor isoforms, Notch1 and Notch2 are expressed in biliary epithelium.[25] Persistence of Hairy/enhancer of split-1 (Hes-1), a downstream target of Notch pathway activation in the livers of BA patients at the time of KPE, suggests that Notch signaling may play a role in the pathogenesis of BA.[26] Thus, we hypothesize that activated Notch signaling is associated with ductular reactions within biliary fibrosis in cholestatic liver diseases such as BA.

Herein, we demonstrate evidence of Notch pathway activation in association with ductular reactions in the livers of patients with BA and an experimental model of cholestasis in mice.

Methods

Mouse model of BA

Prom1-cre transgenic mice (Jackson Laboratory, Bar Harbor, ME), in which a knocked-in Cre recombinase transgene disrupts the *Prom1* gene locus, were bred.[7] Thus, *Prom1*-cre$^{+/+}$ mice are functionally *Prom1* knockouts (KO); hence, *Prom1*-cre$^{-/-}$ littermates are wild type (WT). Six-wk-old adult mice were fed 3,5-diethoxycarbonyl-1,4-dihydrocollidine (DDC) dietary regimen *ad libitum* for 2 wk, at which time serum and liver were analyzed for bilirubin level, gene expression (quantitative polymerase chain reaction [qPCR], Western blot) and histology (immunofluorescence [IF], Sirius red, H&E). All animal experiments were in accordance with protocols approved by the Institutional Animal Care and Use Committee of Children's Hospital Los Angeles (CHLA).

IF staining

Livers were fixed in 4% paraformaldehyde (Polysciences, Inc, Warrington, PA) and embedded in paraffin or optimum cutting temperature (Sakura Finetek, Torrance, CA) for sectioning. IF staining was performed as described previously.[27] Signals were detected by secondary antibodies conjugated either with anti-mouse cyanine Cy3/Cy5, anti-rat Cy3/Cy5, or anti-rabbit Cy3/Cy5 (Jackson ImmunoResearch Labs, West Grove, PA). Fluorescence images were acquired using a Leica DM5500B IF microscope using Leica Suite Advanced Fluorescence 6000 software (Leica Microsystems, Wetzlar, Germany). Bright-field images were acquired using a Leica DM1000 (DFC290) transmitted light microscope (Leica Microsystems AG, Heerbrugg, Switzerland) with the Leica Application Suite (version 2.7.1R1).

Immunohistochemistry staining

Staining was performed as previously reported.[28] Briefly, 5-μm thick paraformaldehyde-fixed, paraffin-embedded liver sections were treated with 0.3% methanol/hydrogen peroxide to block endogenous peroxidases. Slides were then washed in phosphate-buffered saline and then microwaved in 50% Tris-EDTA for antigen retrieval. Once cooled on ice, slides were then washed with Tris-buffered saline (TBS) Triton 0.5% followed again with phosphate-buffered saline. Nonspecific blocking was accomplished with 5% goat serum followed by incubation with primary antibody in 5% goat serum at room temperature for 1 h. Slides were then washed with TBS and incubated with secondary antibody (Histostain-Plus IHC HRP Kit [Invitrogen, Carlsbad, CA]) for 40 min. Slides were again washed with TBS and then enzyme conjugate added followed by substrate chromagen mixture as per protocol.

Human BA tissue analysis

Human biopsy samples and relevant clinical data were collected from the Biliary Atresia Research Consortium database (http://genet.cchmc.org), as well as the Human Liver Tissue Library at CHLA. These analyses were in accordance with a protocol approved by the Institutional Review Board of CHLA.

Quantitative polymerase chain reaction

Total RNA was isolated from snap-frozen mouse liver tissues. Complementary DNA synthesis, reverse-transcriptase polymerase chain reaction (RT-PCR), and quantitative real-time