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ORIGINAL RESEARCH—BASIC

Hepatic Aquaporin 10 Expression Is Downregulated by Activated NFκB Signaling in Human Obstructive Cholestasis

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BACKGROUND AND AIMS: Recent studies reported that the hepatic expression of AQP8 and AQP9 was downregulated in bile duct-ligated (BDL) rats and that overexpression of human AQP1 in the rat liver attenuated cholestasis. However, the hepatic expression of AQP10 and its regulatory mechanism in human cholestasis remain unclear. **METHODS:** Serum and liver samples were collected from 34 patients with obstructive cholestasis and from 12 control patients. Eight-week-old male C57BL/6J mice were intravenously injected with an adeno-associated virus 8 (AAV8) encoding human AQP10 driven by a hepatocyte-specific *Alb* promotor (AAV8-*Alb* promotor-*hAQP10*) for functional studies. Constructs of the AQP10 promoter and PLC/PRF/5-ASBT cell lines were used for regulatory mechanism studies. **RESULTS:** AQP10 was significantly downregulated in patients with obstructive cholestasis and negatively associated with the serum levels of total bile acid (TBA). The hepatocyte-specific overexpression of *hAQP10* significantly attenuated the cholestatic liver injury and intrahepatic bile acids (BA) accumulation in BDL mice. Conjugated BAs, such as TCA and inflammatory factor TNFα, significantly repressed AQP10 expression. Furthermore, NFκB p65/p50 directly bound to the AQP10 promotor and decreased its activity in PLC/RPF/5-*ASBT* cells and in the livers of patients with obstructive cholestasis. However, these changes were diminished by BAY 11-7082 (a specific inhibitor of NFκB signaling). **CONCLUSION:** We are the first to report that AQP10 was significantly decreased in patients with obstructive cholestasis. AQP10 overexpression significantly attenuated cholestatic liver injury in BDL mice. Therefore, overexpression of *hAQP10* in the liver may be a valuable strategy for cholestasis intervention.

Keywords: Aquaporin; Adeno-Associated Virus; Cholestasis; Nuclear Factor-Kappa B (NFκB)

Introduction

Impaired bile formation and secretion lead to cholestasis, which is characterized by excessive accumulation of bile acids (BAs) in the liver and serum.[1] Most studies have revealed adaptive responses to cholestasis, including repression of BA synthesis (eg, CYP7A1 downregulation) and enhancement of BA efflux (eg, MRP3, MRP4, OSTα/β, and OATP3A1 upregulation).[2,3] Notably, water accounts for 95% of bile and aquaporins (AQPs) are crucial for bile formation.[4,5] However, the functional and regulatory mechanisms of AQPs in human cholestasis are still largely unclear.

AQP water channels are small integral membrane proteins that are responsible for water transport in the epithelium.[1] At present, 13 AQP isoforms (AQP0-AQP12) have been identified in humans,[6–8] which are divided into 3 subfamilies, namely, the classical AQPs, aquaglyceroporins and unorthodox AQPs.[9] AQPs are widely expressed in various tissues and have species and tissue specificity.[9] Moreover, AQP0, AQP8, and AQP9 are expressed in rat hepatocytes, while AQP1 is expressed in cholangiocytes, gallbladder epithelial cells, and peribiliary vascular endothelial cells.[10–12] Previous studies have shown that downregulated AQP1 and AQP8 expression is associated with the development of idiopathic intrahepatic and bile duct ligation (BDL)–induced extrahepatic cholestasis due to their impairment of bile flow.[13,14] In addition, AQPs were significantly decreased in the liver during sepsis.[15] Lehmann

***These authors contributed equally to this study and shared the first authorship.**

Abbreviations used in this paper: **AQP, aquaporin; BA, bile acid; CA, Cholic acid; CDCA, chenodeoxycholic acid; ChIP, chromatin Immunoprecipitation; DMSO, deoxycholicacid dimethyl sulfoxide; GCA, glycocholate acid; GCDCA, Glycochenodeoxycholic Acid; IF, immunofluorescence; IHC, immunohistochemistry; NPA, asparagine-proline-alanine; TBA, total bile acids; TCA, taurocholic acid; TCDCA, Taurochenodeoxycholic acid; TDCA, taurohyodeoxycholic acid.**

Most current article

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et al[16] injected lipopolysaccharide (LPS) into rats to study the function of AQP8 in LPS-induced cholestasis and found that LPS induces the downregulation of AQP8 expression in hepatocytes and aggravates cholestasis. Furthermore, downregulated AQP9 expression in the hepatocyte basolateral plasma membrane is related to extrahepatic cholestasis.[17] Previous studies have shown that AQP1, AQP3, AQP7, AQP8, AQP9, and AQP10 are expressed in the human liver[18–20]; AQP3 and AQP7 are involved in glycerol transport mediated by insulin[18]; AQP9 is involved in basolateral water transport in hepatocytes[17]; and AQP8 is closely related to canalicular membrane water permeability, while AQP1 is expressed in smaller proliferating bile ducts.[13,21] However, the function of AQP10 in the liver has not been clarified. AQP10, an aquaglyceroporin, is expressed selectively in the human duodenum and jejunum and functions in the transport of water and glycerol,[22] and interestingly, it has been reported that the expression of AQP10 is inhibited in differentiated human adipocytes, resulting in a 50% decrease in water permeability.[23] However, there is no information about its regulatory mechanism in obstructive cholestasis.

In the present study, we aimed to investigate the expression of hepatic AQP10 and its regulatory mechanism in human obstructive cholestasis. Our findings first revealed that AQP10 expression was significantly decreased in the livers of patients with obstructive cholestasis. The hepatocyte-specific overexpression of human AQP10 significantly attenuated cholestatic liver injury and intrahepatic BA accumulation in BDL mice. Overexpression of AQP10 in the liver may be a valuable strategy for intervention in cholestasis.

Material and Methods

Ethical Permission

This research was carried out as per the Declaration of Helsinki (as revised in 2013) published by the World Medical Association and was approved by the Institutional Ethics Review Board of the First Affiliated Hospital of Army Military Medical University (No.KY2021115). A written informed consent was obtained from all patients.

All animal experiments were performed as per the guidelines of the animal care and use committees at the medical research center and the study protocol was approved by the institutional Animal Use and Care Committee of Southwest Hospital affiliated with the Third Military Medical University (No. AMUWEC20201548).

Patients and Liver Sample Collection

All liver tissues were collected from the Biobank of the First Affiliated Hospital of Army Military Medical University, a total of 34 surgical cholestatic liver samples were collected from patients with a pancreatic or periampullary malignancy, control liver samples were obtained from patients undergoing resection of liver metastases without cholestasis. Those patients did not receive ursodeoxycholic acid or any preoperative therapies. The liver samples were cut into small pieces and fixed in 4% paraformaldehyde or snap-frozen in liquid nitrogen. The demographic and clinical characteristics of these subjects are shown in Table A1.

Adenoviral Vectors

AAV8 (*Alb* promotor -*hAQP10* -T2A -Luciferase-40 PolyA) was purchased from Shanghai GeneChem. A schematic representation of the AAV8-*hAQP10* vector genome, consisting of an expression cassette including a liver-specific albumin promoter, the *hAQP10* coding sequence, T2A coding sequence, a luciferase protein coding sequence, and SV40 PolyA flanked by adeno-associated virus inverted terminal repeats, is shown in Figure 1A.

Experimental Animals

All mice were housed under a 12-hour light/12-hour dark cycle and had free access to water and food. Male C57BL/6J mice were purchased from GemPharmatech Co, Ltd (Nanjing, China). Eight-week-old mice (C57BL/6J) were randomly divided into 4 groups for the BDL experiments or the sham operation (sham-Luc [7 days, n = 5], sham-*hAQP10* [7 days, n = 5], BDL-Luc [7 days, n = 5], BDL-*hAQP10* [7 days, n = 5]), and received a single administration of either AAV8-*hAQP10* or AAV8-Luc via tail vein injection [1×10^{11} vector genomes v.g.]. The animals were imaged at 2 weeks after the injection of AAV8, and after another 2 weeks, all mice were subjected to the BDL or sham operation for 7 days; surgery was performed under anesthesia with ketamine (Nimatek, 100 mg/kg) and xylazine (Sedamun, 10 mg/kg). The mice were fasted for 12 hours before they were sacrificed. Serum was collected and stored at −80 °C and liver tissues were frozen in liquid nitrogen. Serum and liver tissue biochemical assays were performed by the clinical laboratory at Southwest Hospital affiliated with the Third Military Medical University (Chongqing, China). Successful adenovirus-mediated liver-specific expression of the AQP10 gene was analysed by quantitative real-time polymerase chain reaction (qRT-PCR) and immunohistochemistry (IHC) (Figure 1C and D).

Adenoviral Vectors

AAV8 (*Alb* promotor -*hAQP10* -T2A -Luciferase-40 PolyA) was purchased from Shanghai GeneChem. A schematic representation of the AAV8-*hAQP10* vector genome, consisting of an expression cassette including a liver-specific albumin promoter, the hAQP10 coding sequence, the thosea asigna virus 2A (T2A) coding sequence, a luciferase protein coding sequence and SV40 PolyA (Simian virus 40 PolyA, also called PolyA) flanked by adeno-associated virus inverted terminal repeats, is shown in Figure 2A.

Animal Imaging

All liver tissues expressing the AQP10 gene expression sequence were detectable because of the AAV8-mediated expression of the luciferase gene. These mice were anaesthetized using 1.5% isoflurane and intraperitoneally injected with 10 μL of a D-luciferin stock solution (MCE, HY-12591B China) per gram of body weight. Photon emission from the region of interest (entire animal) was measured using the

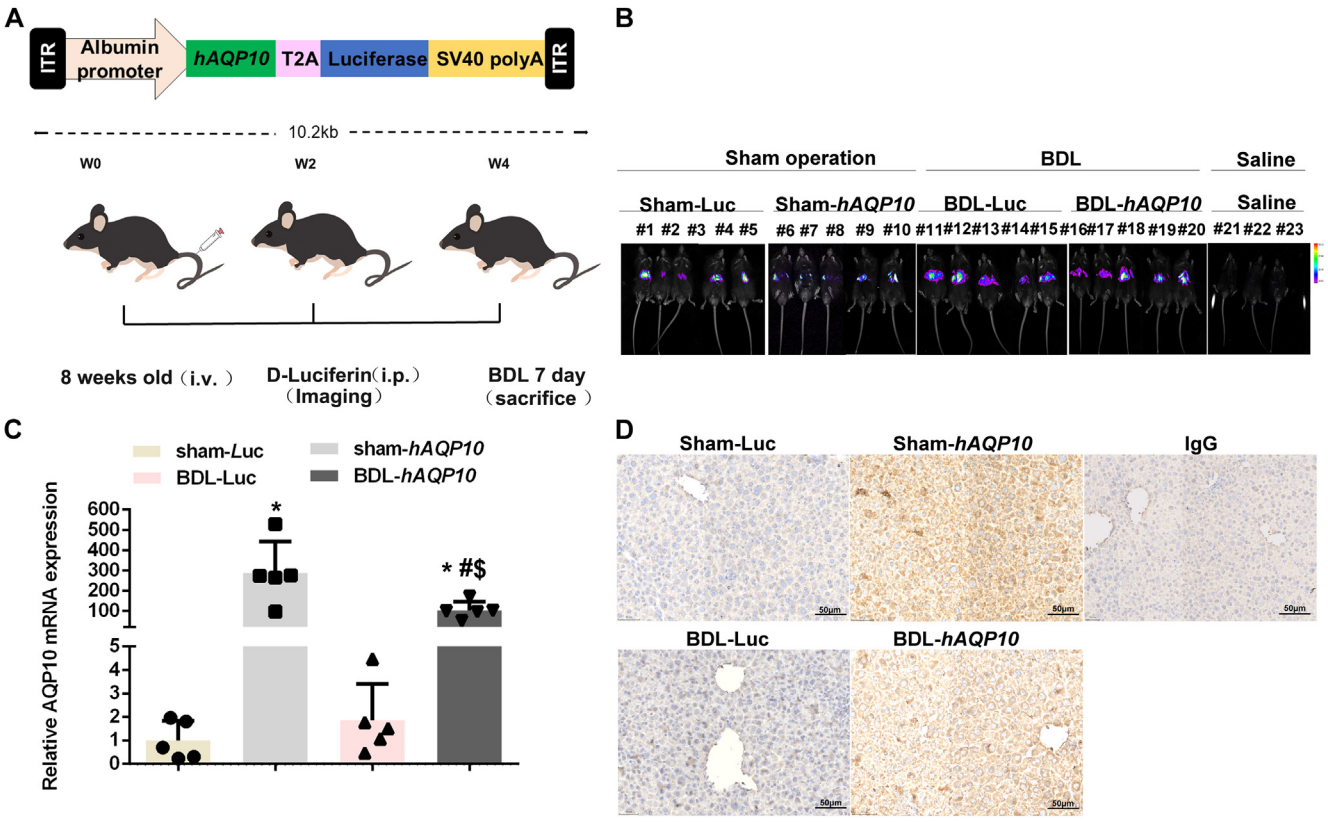

Figure 1. Design and in vitro validation of AAV8-mediated liver *hAQP10* gene transfer. (A) Schematic representation of the vector expression cassette and experimental design. (B) A representative image of AAV8-mediated *hAQP10* gene transfer in mice. (C) AQP10 mRNA expression in AAV8-mediated livers (sham-Luc, n = 5; sham-*hAQP10*, n = 5; BDL-Luc, n = 5; BDL-*hAQP10*, n = 5). $^{*}P < .05$ vs the sham-Luc group. $^{\#}P < .05$ vs sham-*hAQP10*. $^{\$}P < .05$ vs BDL-Luc. (D) Immunohistochemistry (IHC) labelling of *hAQP10* in the livers of AAV8-mediated *hAQP10* gene transfer mice. The *hAQP10* expression was significantly increased in the sham-*hAQP10* group and BDL-*hAQP10* group.

device IVIS Imaging System 100 (Xenogen/Caliper Life Sciences, Alameda, California).

Cell Culture and Treatment

Human hepatoma PLC/RPF/5-*ASBT* cells and PLC/RPF/5 cells were maintained in our laboratory.[3] The cells were cultured in DMEM (Gibco) containing 10% fetal bovine serum (FBS, complete medium, HyClone) at 37 °C and 5% CO_2. When the cells grew to 50% confluency, they were treated with vehicle dimethyl sulfoxide (DMSO), 100 μM cholic acid (CA), chenodeoxycholic acid (CDCA), glycocholate acid (GCA), glycocholic acid (GCDCA), taurocholic acid (TCA), taurochenodeoxycholic acid (TCDCA), and taurohyodeoxycholic acid (TDCA, Sigma) and cultured in DMEM containing 1% charcoal stripped FBS (CS-FBS, Gibco) for 12 hours. In some experiments, the cells were pretreated with 10 μM BAY 11-7082, an NFκB inhibitor, for 30 minutes.

Plasmid Construction, Transfection, and Generation of Stably Transfected Cell Lines

PLC/RPF/5-*ASBT* and PLC/RPF/5 cells (10^6 cells/well) were cultured in complete medium in 6 well plates overnight and transfected with 2 μg of control pcDNA3.1 or pcDNA3.1-AQP10, which we constructed previously. The cells were treated with G418 (750 μg/mL) and puromycin (2 μg/mL) for 21 days to establish cell lines stably expressing the target gene to thereby measure the concentration of bile acid.

AQP10 Promoter Luciferase Reporter Assays

The AQP10 proximal promoter (−1812 to +79) and its truncated forms (−1812, −1660, −877, −434, or −228 to +79) were amplified by PCR using specific primers (Figure 3) and cloned into pGL3 to generate different AQP10 proximal promoter luciferase reporter constructs, followed by sequencing. PLC/RPF/5-*ASBT* cells (1×10^5 cells/well) were cultured in 48 well plates overnight and transfected with phRL-CMV (2.0 ng) and 100 ng of the individual luciferase reporter constructs together with pcDNA3.1-NF-κBp65 and pcDNA3.1-NF-κBp50 (kindly provided by Dr Cai, School of Medicine, Yale University) or control pcDNA3.1 using Fugene HD transfection reagent (Promega, Wisconsin). At 36-hour post-transfection, the cells were treated with DMSO or TCA (100 μM) for 24 hours and lysed in lysis buffer. The luciferase activity of individual groups of cells was measured using the Dual Luciferase Assay kit (Promega) as per the manufacturer's instructions.

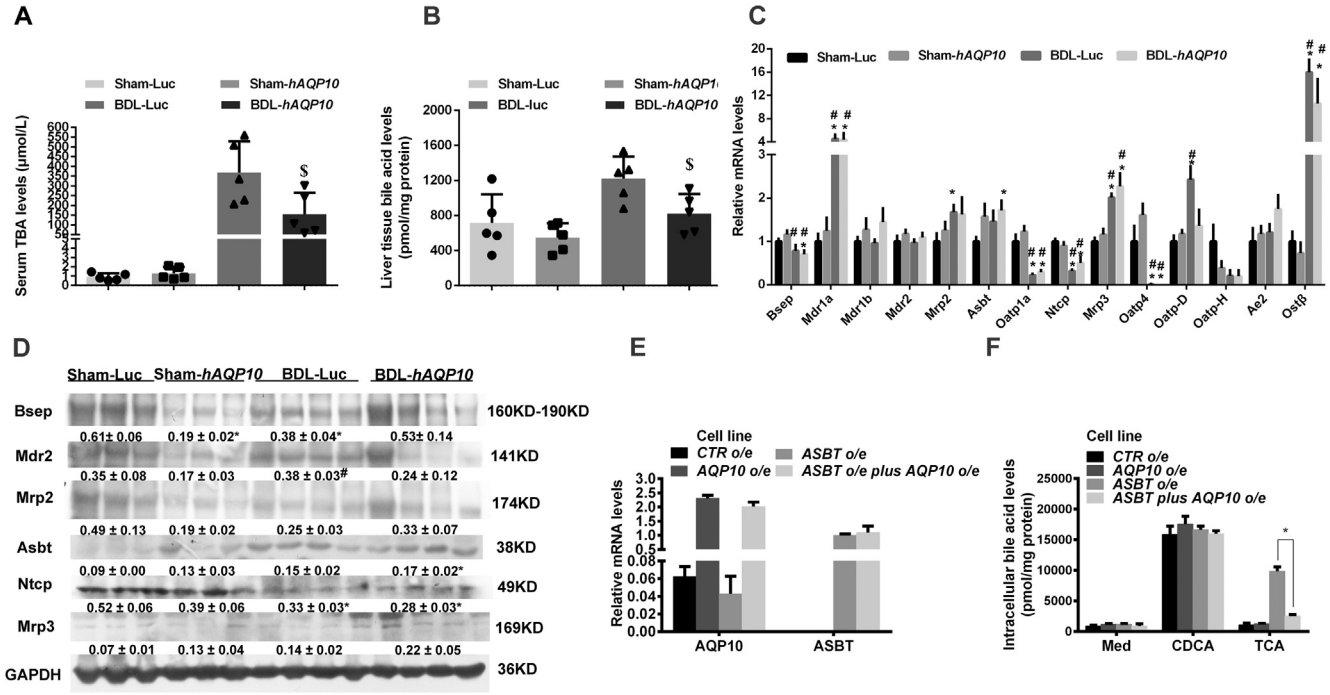

Figure 2. Hepatocyte-specific overexpression of human AQP10 significantly reduced the cholestatic liver injury and intrahepatic BA accumulation in BDL mice and TCA-treated hepatoma PLC/PRF/5-*ASBT* cells. (A) Serum TBA levels in mice, $^{\$}P < .05$ vs BDL-Luc. (B) Liver tissues of the same weight were collected for ultrasonication and samples were analysed at the clinical laboratory of Southwest Hospital affiliated with Army Medical University, $^{\$}P < .05$ vs BDL-Luc. (C) The mRNA expression of hepatic transporters. The decreased levels hepatic and serum bile acids were not due to hepatic transporters. $^{*}P < .05$ vs Sham-Luc, $^{\#}P < .05$ vs Sham-*hAQP10*. (D) Representative Western blot images of Mrp2, Mdr2, Mrp3, Asbt, Ntcp, and Bsep, $^{*}P < .05$ vs Sham-Luc, $^{\#}P < .05$ vs Sham-*hAQP10*. (E) qRT-PCR analysis of the AQP10 and ASBT expression in PLC/PRF/5-*Ctrl*, PLC/PRF/5-*ASBT*, PLC/PRF/5-*AQP10*, and PLC/PRF/5-*ASBT-AQP10* cells. (F) AQP10 overexpression reduces the accumulation of bile acids in human hepatoma cells. PLC/PRF/5-*Ctrl*, PLC/PRF/5-*ASBT*, PLC/PRF/5-*AQP10*, and PLC/PRF/5-*ASBT-AQP10* cells were treated in triplicate with DMSO, control CDCA, or TCA for 12 h. $^{*}P < .05$ vs the ASBT o/e.

Quantitative Real-Time Polymerase Chain Reaction

Total RNA was extracted from tissues or cultured cells using TRIzol reagent (Invitrogen, San Diego, California) and reverse transcribed into first-strand cDNA. The transcript levels of the target genes relative to that of the control GAPDH mRNA were determined by qRT-PCR using SYBR Premix Ex TaqTM Green II (Takara, Japan) and specific primers (Table A3) on a real-time PCR system (BioRad). The data were normalized to the control and analysed by $2^{-\Delta\Delta Ct}$ using SDS software (Applied Biosystems).

Western Blot Analysis

The cell lysate samples were prepared as described previously[24] and resolved by sodium dodecyl sulfate polyacrylamide gel electrophoresis, followed by transfer onto polyvinylidene difluoride membranes. The membranes were blocked with 5% fat-free dry milk in tris-buffered saline with 0.5% Tween 20 and incubated with primary antibodies, as shown in Table A4. In particular, we used an affinity-purified rabbit polyclonal antibody against the human AQP10 protein (Alpha Diagnostic, San Antonio; catalogue number AQP10 1-S/A; lot number 674142S). After being washed, the bound antibodies were detected with horseradish peroxidase–conjugated secondary antibodies and visualized using enhanced chemiluminescent reagents. The levels of the target proteins relative to those of the control GAPDH were analysed by ImageJ software.

Chromatin Immunoprecipitation Assays

The potential binding sequences of NFκB in the chromosome were determined by chromatin immunoprecipitation (ChIP) assays performed using a commercial ChIP Assay Kit (Millipore, Bedford, Massachusetts). Briefly, PLC/PRF/5-*ASBT* cells were cultured, and upon reaching 80% confluence, they were pretreated with the vehicle DMSO or 10 μM BAY 11-7082 for 30 minutes. The cells were treated with vehicle or 100 μM TCA for 24 hours. Soluble chromatin was prepared and then immunoprecipitated using antibodies against NFκB p65 (Table A4). The primer sequences and the sizes of the amplicons are listed in Table A6. In addition, human liver tissues from 4 controls and 5 patients with cholestatic disease were used for the preparation of soluble chromatin.

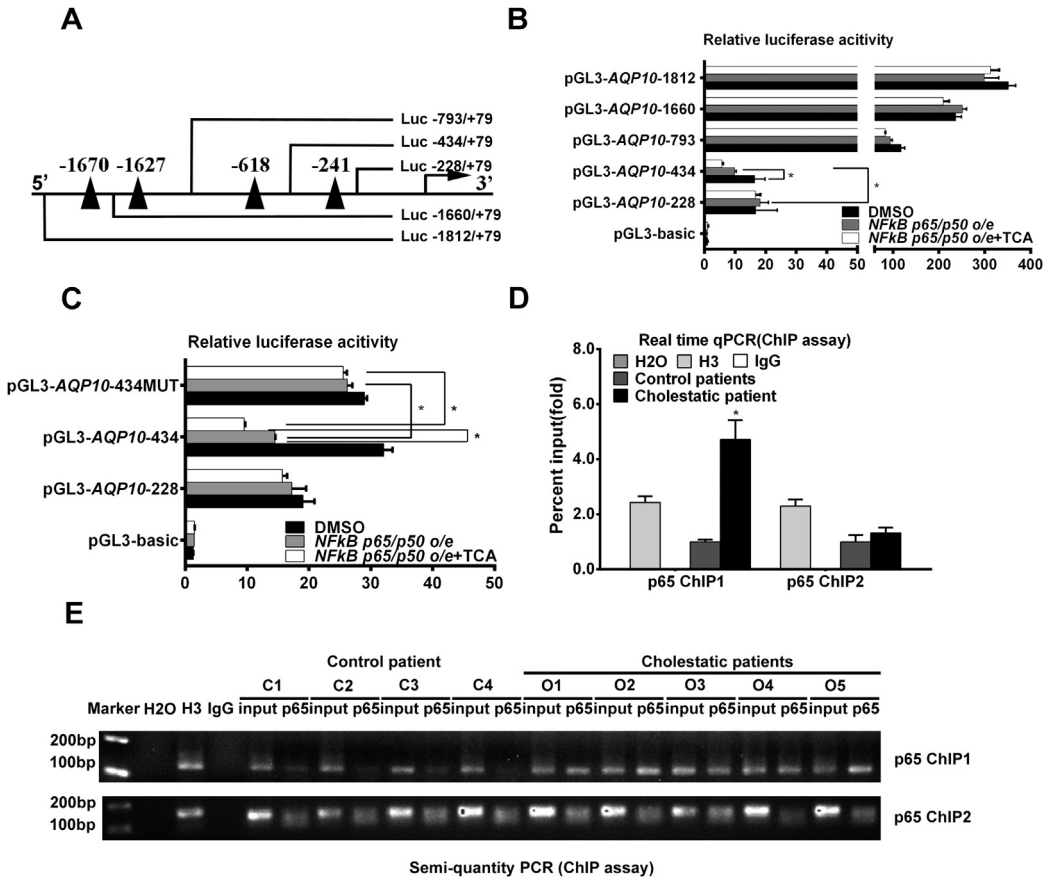

Figure 3. The transcription factor NFκB p65 directly binds to the AQP10 promotor and decreases its activity in human obstructive cholestatic livers. (A) Illustration of potential NFκB response regions in the AQP10 promoter. (B) Luciferase assay analysis of the binding of NFκB p65 to the AQP10 promoter and its truncate and the influence of TCA. PLC/PRF/5-ASBT cells were cotransfected with a control plasmid and individual truncates together with or without plasmids encoding NFκB p65 and NFκB p50 expression and treated with DMSO or TCA for 24 h. The luciferase activities were measured. (C) NFκB p65/p50 overexpression and/or TCA treatment significantly decreased the AQP10 −434 to +79 truncate-controlled luciferase activity but not the mutant-controlled luciferase activity, in PLC/PRF/5-ASBT cells. PLC/PRF/5-ASBT cells were cotransfected with the AQP10 promoter −434 or −228 truncate together with or without plasmids encoding NFκB p65 and NFκB p50 expression and treated with DMSO or TCA for 24 h. The luciferase activities in individual groups of cells were measured. *$P < .05$ vs the control group. (D) ChIP/qRT-PCR analysis of NFκB p65 binding to the AQP10 promoter using specific primers in human liver samples. *$P < .05$ vs the control patients. (E) ChIP-semiquantitative PCR analysis of NFκB p65 binding to the AQP10 promoter in the livers of obstructive cholestatic patients.

Determination of the Bile Acid Concentrations in Liver Tissues and Cells

Snap-frozen liver samples of equal weights were homogenized in 1 mL of precooled phosphate buffer solution, sonicated, and centrifuged. The bile acid contents in individual samples were determined by the clinical laboratory at Southwest Hospital affiliated with Army Medical University (Chongqing, China). The different groups of cells were treated with DMSO or 100 μM TCA and GCA for 24 hours. After being washed, the cells were lysed with 500 μL of 1% Triton X-100 and sonicated, followed by centrifugation. After quantifying the protein concentrations, the levels of bile acid in individual samples were determined by the clinical laboratory.

Immunofluorescence and Immunohistochemistry Analysis

The snap-frozen liver tissue samples were fixed with 4% paraformaldehyde, dehydrated by 30% sucrose, and embedded in O.C.T compound. Crystal tissue sections were prepared and the expression of AQP10 in the liver tissues and hepatoma cells was characterized by immunofluorescence and IHC, as previously described,[25] using the primary antibodies in Table A4.

Statistical Analysis

All data are expressed as the mean ± standard deviation. The independent data were analysed by Student's *t*-test (2-tailed) using GraphPad Prism 6.0. An average value of $P <$ .05 was considered statistically significant.

Results

Hepatic AQP10 Expression Was Significantly Reduced in Patients With Obstructive Cholestasis

To determine the expression of AQP10 in the livers of patients with cholestasis, 34 surgically obtained

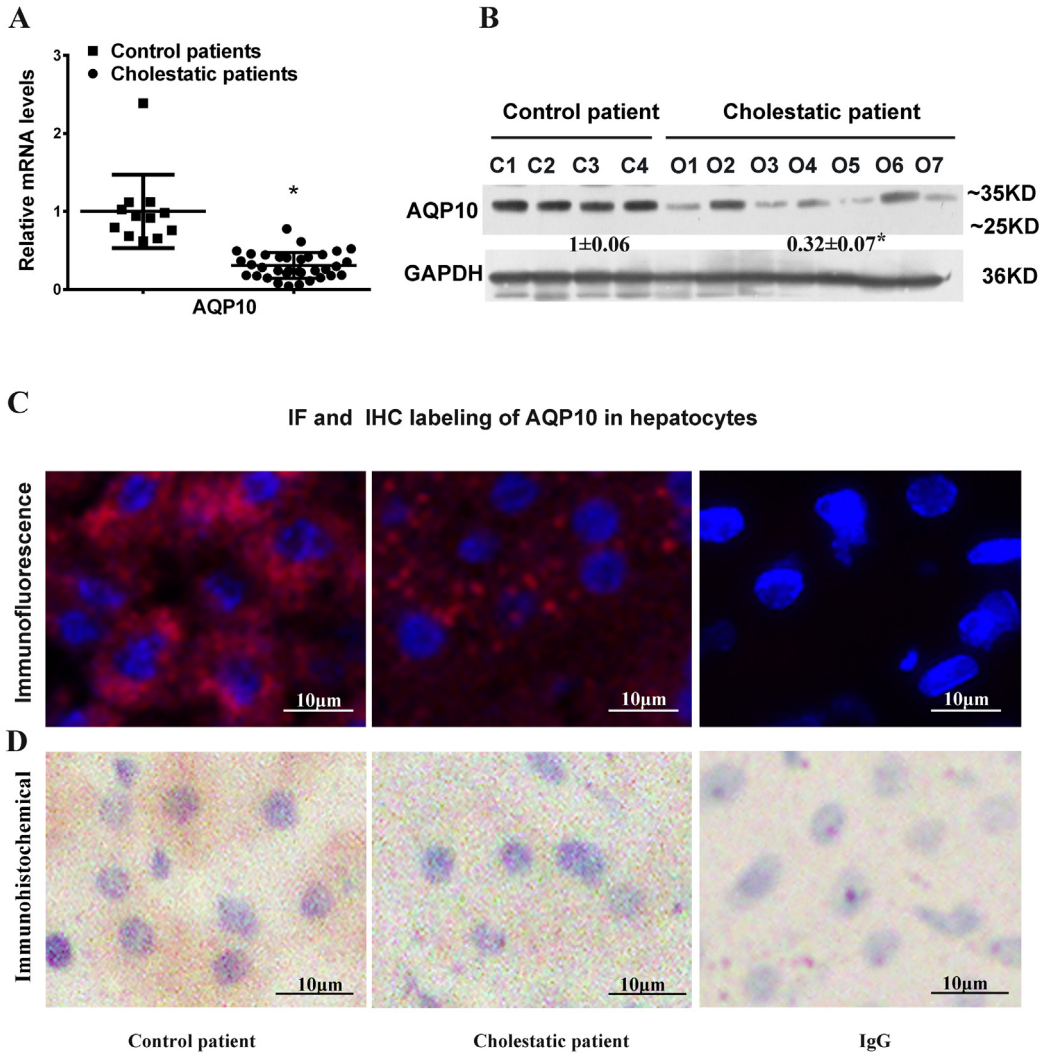

Figure 4. AQP10 expression was significantly reduced in the human obstructive cholestatic liver. (A) AQP10 mRNA transcripts in human livers (relative to the control group; n = 12 for the control group, n = 34 for the obstructive cholestatic group). *$P < .0001$ vs controls. (B) Western blot analysis of the relative levels of AQP10 expression in human liver samples (C1–4: control patients; O1–O7: obstructive cholestasis patients). *$P < .0001$ vs controls. (C) Immunofluorescence staining of the AQP10 protein (red) in liver samples from a control patient and a patient with obstructive cholestasis. (D) Immunohistochemistry analysis of AQP10 expression. Immunohistochemistry labelling of AQP10 in the liver of a control patient and a patient with obstructive cholestasis. AQP10 expression was reduced in the liver of the obstructive cholestasis patient.

cholestatic liver samples from patients with pancreatic or periampullary malignancy and 12 liver samples from patients with liver metastatic tumours were obtained. As expected, the liver function and bile acid metabolism were abnormal in patients with cholestatic livers (Table A1). The results indicated that the relative mRNA levels of AQP10 were notably reduced in cholestatic patients by 3.3 fold and that the protein expression in the cholestatic livers was 3.1-fold lower than that in control patients (Figure 4A and B). Immunofluorescence and IHC analysis indicated that the AQP10 expression was markedly reduced in the liver tissues of cholestatic patients (Figure 4C and D).

Observance of a Significant Negative Correlation Between the Serum Levels of Total Bile Acids and Hepatic mRNA AQP10 Expression in Obstructive Cholestatic Patients

To investigate the relationship between AQP10 and cholestasis, we performed a correlation analysis, revealing that the relative mRNA levels of AQP10 were inversely correlated with the levels of serum total bile acids (TBA) ($r = -0.3648$, $P = .0339$, Figure 5F) but not with the levels of alanine aminotransferase, aspartate aminotransferase, γ-glutamyl transferase, alkaline phosphatase, and total bilirubin in patients with cholestatic liver in this population. Therefore,

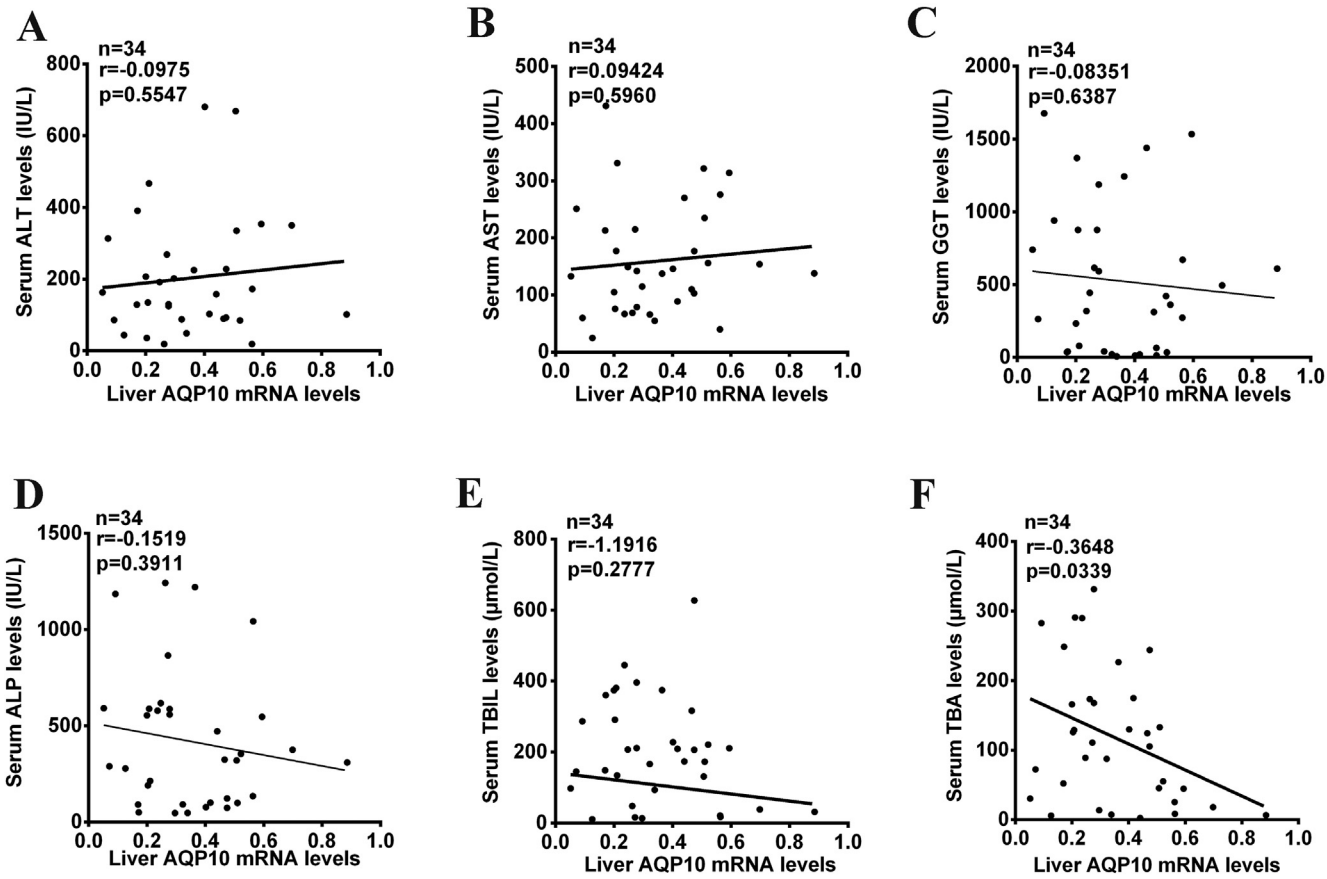

Figure 5. (A–F) Downregulated AQP expression is inversely correlated with abnormal bile metabolic parameters in patients with cholestatic diseases. AQP10 mRNA transcript levels were inversely correlated with the levels of serum TBA ($r = -0.3648$, $P = .0339$, $n = 34$), whereas no significant association was observed between the AQP10 mRNA transcript levels and the serum AST, ALT, ALP, GGT, and TBIL levels.

the hepatic AQP10 expression was significantly reduced in obstructive cholestatic patients, and downregulated AQP10 expression was inversely correlated with abnormal bile acid metabolism in patients with cholestatic liver diseases.

Generation and Validation of the Hepatocyte-Specific Overexpression of Human AQP10 in Mice by the AAV8-Alb Promoter-hAQP10 Construct

To further study the role of AQP10 in cholestasis, we purchased a vector from Shanghai GeneChem (AAV8-mediated *hAQP10* gene), and a schematic representation of the vector expression cassette and the experimental design is shown in Figure 1A. The adenovirus was injected into mice through the caudal vein at 8 weeks, and the amounts of the adenovirus-mediated genes delivered are detailed in the methods section. After 2 weeks, all mice were imaged in vivo (Figure 1B); after another 2 weeks, the animals were subjected to the BDL or sham operation. As shown in Figure 1C and D, qRT-PCR and IHC revealed that the AQP10 expression was notably increased in the sham-*hAQP10* group and BDL-*hAQP10* group.

Hepatocyte-Specific Overexpression of Human AQP10 Significantly Reduced the Cholestatic Liver Injury and/or Intrahepatic BA Accumulation in BDL Mice and in TCA-Treated Hepatoma PLC/PRF/5-ASBT Cells

The TBA levels in serum or liver tissue were markedly lower in the BDL-*hAQP10* group than in the BDL-Luc group, while no obvious effects were observed in noncholestatic control mice ($P < .05$; Figure 2A and B). Solute membrane transporters are important pathways of bile transport; to our surprise, the levels of the liver transporters Bsep, Mdr1a, Mdr1b, Mdr2, Mrp2, Asbt, Oatp 1a, Ntcp, Mrp3, Oatp4, Oatp-D, Oatp-H, Mrp4, Ostβ and Ae2 mRNA levels did not differ between the BDL-Luc group and the BDL-*hAQP10* group, the levels of the liver transporters Bsep, Mdr2, Mrp2, Asbt, Ntcp, Mrp3 protein levels also did not differ between the BDL-Luc group and the BDL-*hAQP10* group (Figure 2C and D). To determine the role of AQP10 in cholestasis, PLC/PRF/5 and PLC/PRF/5-*ASBT* cells were transfected with the pcDNA3-AQP10 plasmid or the control plasmid to establish stable PLC/PRF/5-*Ctrl*, PLC/PRF/5-*ASBT*, PLC/PRF/5-*AQP10*, and PLC/PRF/5-*ASBT-AQP10* cells. AQP10 and ASBT expression

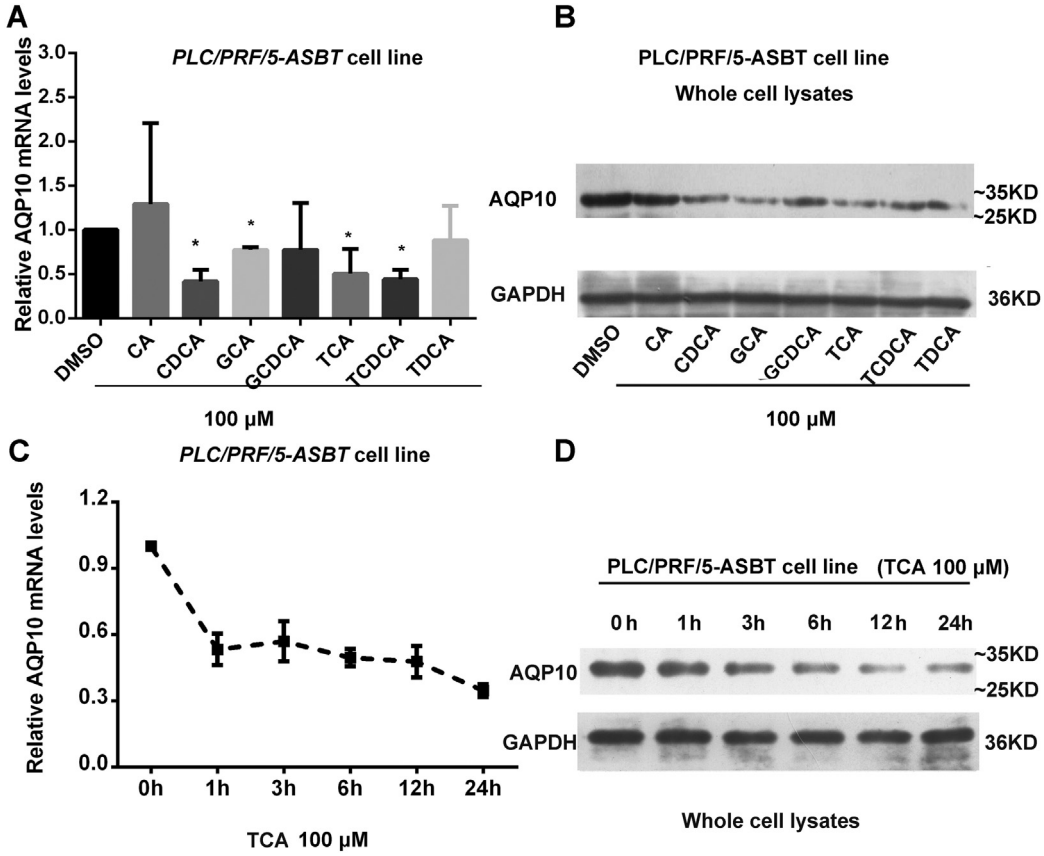

Figure 6. Bile acids decreased the AQP10 mRNA and protein expression in PLC/PRF/5-ASBT cells in a time-dependent manner. (A and B) qRT-PCR and Western blot showed that the AQP10 expression was significantly decreased in PLC/PRF/5-*ASBT* cells treated with the conjugated bile acids CDCA, GCA, TCA, TCDCA, and TDCA. *$P < .05$ vs the control group. (C) Time-dependent inhibition of AQP10 mRNA transcription. (D) Time-dependent inhibition of AQP10 protein expression.

was detected by Western blot (Figure 2E). Treatment with DMSO or 100 mM CDCA did not alter the levels of bile acids in the different groups of cells (Figure 2F). However, while basal levels of bile acids were detected in PLC/PRF/5-Ctrl and PLC/PRF/5-AQP10 cells, the levels of bile acids in PLC/PRF/5-*ASBT-AQP10* cells were significantly lower than those in PLC/PRF/5-*ASBT* cells ($P < .05$).

Together, our findings suggest that the overexpression of human AQP10 can attenuate BA accumulation in hepatocytes.

Bile Acids Decreased the mRNA and Protein Expression of AQP10 in Hepatoma PLC/PRF/5-ASBT Cells in a Time-Dependent Manner

Given the negative correlation between AQP10 and abnormal bile acid metabolism, we examined whether bile acids could modulate AQP10 expression in cholestatic hepatocytes. PLC/RPF/5-ASBT cells were treated with vehicle DMSO, CA, CDCA, GCA, GCDCA, TCA, TCDCA, or TDCA, and the relative expression levels of AQP10 were determined by qRT-PCR. Treatment with either CDCA, GCA, TCA, TCDCA, or TDCA significantly decreased the mRNA and protein levels of AQP10, while CA and GCDCA had no effect on these levels ($P < .05$, Figure 6A and B). Furthermore, treatment with TCA for varying amounts of time decreased the mRNA and protein expression of AQP10 in PLC/RPF/5-*ASBT* cells in a time-dependent manner (Figure 6C and D). Thus, our data provide evidence that conjugate bile acid can reduce the AQP10 expression in hepatocytes.

The Transcription Factor NFκB p65 Directly Binds to the AQP10 Promotor and Decreases Its Activity in PLC/RPF/5-ASBT Cells and in the Livers of Patients With Obstructive Cholestasis

To elucidate the molecular mechanisms of AQP10 regulation during cholestasis, we first analysed the AQP10 promoter (1812 bp, http://jaspar.genereg.net) and identified numerous putative NFκB response elements (Figure A2A). To verify which response element(s) were functional, the AQP10 promoter and its truncates (−1812, −1660, −877, −434 and −228 to +79) were cloned into pGL3 to generate different luciferase reporter plasmids (Figure 3A). The luciferase activities controlled by the −434/+79 and −228/+79 truncates were obviously lower than those controlled by the AQP10 promoter and other truncates in PLC/PRF/5-*ASBT* cells, illustrating that the −434 to −228 region of the promoter had vital NFκB

response elements (Figure 3B). Second, treatment with TCA significantly decreased the AQP10 promoter −434/+79 truncate-controlled luciferase activity in PLC/PRF/5-ASBT cells. Furthermore, induction of NFκB p65/p50 overexpression significantly reduced the luciferase activity relative to that of the DMSO control. These results indicated that the key elements for effective NFκB p65/p50 binding were located in the AQP10 promoter between −434 and −228. Additional luciferase assays revealed that the induction of NFκB p65/p50 overexpression significantly decreased the AQP10-434/+79 promoter activity but not the mutant-controlled (site-directed mutation) luciferase activity in PLC/PRF/5-*ASBT* cells (Figure 3C and Figure A2B). To determine whether transcription factors can downregulate AQP10 in cholestatic patients, their expression levels were detected by ChIP-qPCR. The ability of activated NFκB p65 to bind the AQP10 promoter in the −434 to −228 region was significantly increased in patients with cholestatic liver diseases compared with the control patients, and ChIP assays (semiquantitative PCR) further confirmed this result (Figure 3D and E). However, there was no difference in the abilities of activated NFκB p65 to bind to the other tested regions in the AQP10 promoter between the cholestatic patients and controls (Figure A2D). Therefore, these data clearly indicated that NFκB p65 directly inhibited the AQP10 expression in cholestatic livers.

*The Activation of NFκB Signaling by Bile Acids and TNFα Downregulated the AQP10 Expression and the Ability of p65 to Bind the AQP10 Promoter in PLC/RPF/5-*ASBT *Cells*

The activation of NFκB signaling is associated with the development of cholestasis in the liver.[3] TCA is a conjugated bile acid. To understand the molecular mechanisms by which TCA downregulates AQP10 expression, the effect of TCA (100 μM) on NFκB signaling in PLC/PRF/5-*ASBT* cells was assessed for varying amounts of time. We found that TCA increased the relative levels of NFκB p65 phosphorylation in PLC/PRF/5-*ASBT* cells (Figure 7A). Treatment with TCA (100 μM) for 24 h significantly decreased the mRNA levels of AQP10 (Figure 7B). Pretreatment with BAY 11-7082 (10 μM), an irreversible inhibitor of NFκB signaling abrogated the inhibitory effect of TCA on the AQP10 expression in PLC/PRF/5-ASBT cells. Treatment with TNFα (50 ng/ml) in PLC/PRF/5-ASBT cells also decreased the expression of AQP10, and the trend of TNFα downregulating AQP10 expression was reversed by BAY 11-7082 (Figure 7C and D). Moreover, as shown by ChIP assays, the TCA-induced ability of NFκB to bind the AQP10 promoter (NFκB ChIP1) was diminished in the presence of BAY 11-7082 (Figure 7E and F). The TCA-induced ability of NFkB p65 to bind the AQP10 promoter (NFκB p65 ChIP2 and NFκB p65 ChIP3) was not affected by BAY 11-7082 (Figure 7E and Figure A2E).

These data indicated that the activation of NFκB signaling by bile acids and TNFα downregulated the AQP10 expression and the ability of p65 to bind the AQP10 promoter in PLC/RPF/5-*ASBT* cells.

Discussion

Here, we are the first to report that AQP10 was expressed in the human liver and hepatocytes and that its expression was significantly decreased in the livers of patients with obstructive cholestasis. Moreover, we also revealed the regulatory mechanism of AQP10 downregulation in human obstructive cholestasis. Interestingly, hepatocyte-specific overexpression of human AQP10 significantly attenuated cholestatic liver injury and intrahepatic BA accumulation in BDL mice. Therefore, overexpression of AQP10 in the liver may be a valuable strategy for intervention in obstructive cholestasis, while obstructive cholestasis may be very different from cholestasis of other aetiologies, and future research may benefit from examining this possibility in liver biopsies in those settings.

Cholestasis is characterized by the excessive accumulation of intrahepatic BAs.[1] Under cholestasis conditions, adaptive responses, including repression of BA synthesis (eg, CYP7A1 downregulation) and enhancement of BA efflux (MRP3, MRP4, OSTα/β, and OATP3A1 upregulation), are triggered to ameliorate cholestatic liver injury. It is well known that the transport of BAs is closely related to water transport and solute transport, and previous studies have reported that hepatic AQP9 facilitates basolateral water entry, AQP8 and AQP1 enhance solute-driven AQP-mediated biliary water secretion, the hepatic AQP8 and AQP9 expression levels are decreased in cholestatic rats, and overexpression of human AQP1 in rats attenuates cholestasis.[13,17,21] However, the expression of AQP10 in human liver and its functional role in cholestasis remain unclear. Herein, our results confirm the previously reported presence of AQP10 in the human liver (Figure 4); in contrast, it is not expressed in rodent livers.[26] Furthermore, we are the first to report that hepatic AQP10 expression was significantly decreased and negatively correlated with serum total bile acid (TBA) levels in patients with obstructive cholestasis (Figure 5F), indicating that AQP10 may be associated with BA elimination. A recent study demonstrated that the overexpression of human AQP1 improves bile salt secretion in oestrogen-induced cholestatic rats by increasing bile salt export pump (BSEP) transport activity.[21] In the present study, the hepatocyte-specific overexpression of human AQP10 in mice by AAV8-*Alb* promoter-*hAQP10* infection did not alter the hepatic mRNA and protein expression levels of BA transporters such as BSEP, Mrp2, Mrp3, Mdr2 and Ostβ when compared to those of control BDL mice (Figure 2C and D). Nevertheless, *hAQP10* overexpression in hepatocytes significantly reduced BA accumulation in BDL mouse livers and taurocholic acid (TCA)-treated PLC/RPF/5-*ASBT* hepatoma cells (Figure 2A, B, E and F). In addition, the serum levels of biochemistry parameters in BDL mice showed that serum alanine aminotransferase, aspartate aminotransferase, total bilirubin, and direct bilirubin were significantly decreased in the AQP10 overexpression group (Table 1). Therefore, these findings suggest that liver AQP10-targeted therapy can

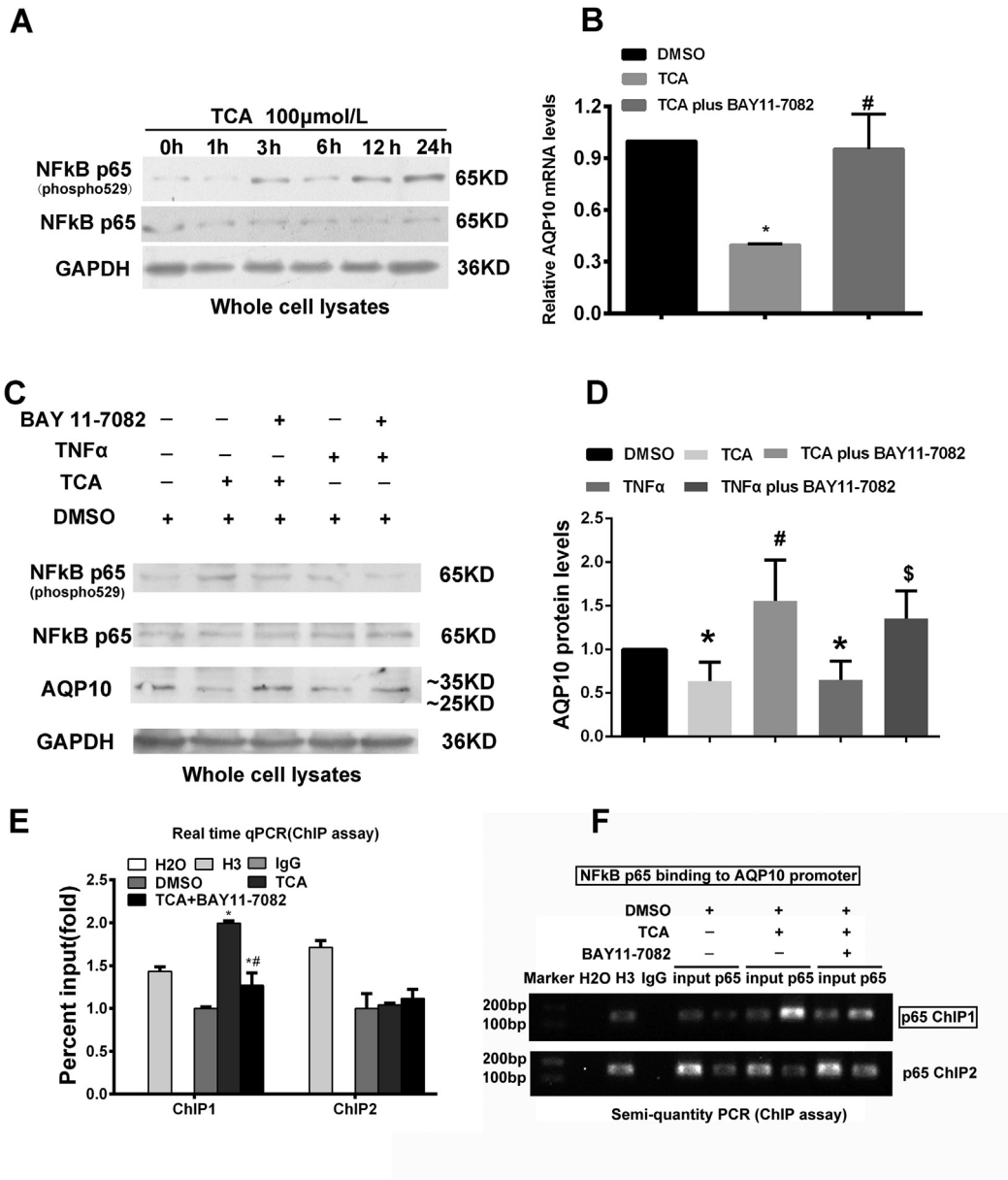

Figure 7. TCA downregulates the AQP10 expression in human hepatocytes by activating NFκB signaling PLC5/PRF/5-*ASBT* cells were treated in triplicate with DMSO and 100 μM TCA for varying amounts of time, pretreated with BAY 11-7082 for 30 min, and treated with DMSO, TCA, or 50 ng/mL TNFα for 24 h. The relative levels of AQP10, NFκB p65, and NFκB p65 phosphorylation were determined by qRT-PCR or Western blot. (A) TCA activates NFκB signaling in PLC5/PRF/5-*ASBT* cells. (B) The levels of AQP10 mRNA transcripts. (C and D) Western blot analysis of AQP10 expression and NFκB activation. The data are representative images or expressed as the mean ± SD of each group from 3 independent experiments. $^{*}P < .05$ vs the DMSO group; $^{\#}P < .05$ vs the TCA group; $^{\$}P < .05$ vs the TNFα group. (E and F) Validation of the ability of NFκB p65 to bind to the AQP10 promoter in PLC5/PRF/5-*ASBT* cells by ChIP/RT-PCR analysis or ChIP semiquantitative PCR analysis. $^{*}P < .05$ vs the DMSO group, $^{\#}P < .05$ vs the TCA group.

reduce BA accumulation and improve AQP10 liver function. The mechanism for reducing BA accumulation may be related to solute transporter activities; however, further investigation need to be carried out in the future.

AQPs, including AQP1, AQP8, and AQP9, play a crucial role in cholestasis.[14,27] However, the regulatory mechanisms of these AQPs, especially AQP10, in human cholestasis are still unknown. Our previous study demonstrated that hepatic NFκB signaling is activated in human obstructive cholestasis.[3] Furthermore, elevated levels of serum TNFα and TBA were observed in patients with obstructive cholestasis[24,28] and recombinant TNFα and conjugated BAs were shown to activate NFκB signaling.[29,30] Similarly, we found that conjugated BAs, such as TCA, stimulated the phosphorylation of NFκB p65 in PLC/RPF/5-*ASBT* hepatoma cells (Figure 7A and B). Further studies revealed that

Table 1. Clinical Features of Bile Duct-Ligated Mice

Clinical features	Sham-Luc (n = 5)	Sham-*hAQP10* (n = 5)	BDL-Luc (n = 5)	BDL-*hAQP10* (n = 5)
ALT (IU/L)	32.97 ± 5.07	27.91 ± 8.60	428.40 ± 48.92[a,b]	221.30 ± 37.41[a,b,c]
AST (IU/L)	151.00 ± 27.86	129.90 ± 21.70	581.00 ± 73.33[a,b]	349.3 ± 53.31[a,b,c]
ALP (IU/L)	99.03 ± 34.38	77.92 ± 9.10	474.90 ± 27.51[a,b]	379.60 ± 37.63[a,b]
TBIL (μmol/L)	0.80 ± 0.31	1.33 ± 0.18	336.10 ± 18.48[a,b]	203.80 ± 50.53[a,b,c]
DBIL (μmol/L)	0.75 ± 0.34	1.69 ± 0.91	255.80 ± 17.25[a,b]	149.50 ± 33.62[a,b,c]
IBIL (μmol/L)	0.46 ± 0.22	0.42 ± 0.28	80.31 ± 18.81[a,b]	54.29 ± 17.80[a,b]
TBA (μmol/L)	0.93 ± 0.18	1.31 ± 0.30	368.60 ± 71.73[a,b]	155.50 ± 48.90[a,b,c]
LDH (IU/L)	566.50 ± 46.10	382.00 ± 35.26	766.20 ± 71.00[b]	576.00 ± 57.04[b]

Serum levels of biochemistry parameters in mice with experimental liver cholestasis induced by bile duct ligation (BDL) for 7 days.
The values are the means ± standard deviations.
Sham-Luc, n = 5; Sham-*hAQP10*, n = 5; BDL-Luc, n = 5; BDL -*hAQP10*, n = 5.
ALP, alkaline phosphatase; ALT, alanine aminotransferase; AST, aspartate aminotransferase; DBIL, direct bilirubin; IBIL, indirect bilirubin; LDH, lactate dehydrogenase; TBA, total bile acids; TBIL, total bilirubin.
[a]$P < .05$ vs Sham-Luc.
[b]$P < .05$ vs Sham-*hAQP10*.
[c]$P < .05$ vs BDL-Luc.

conjugated BAs such as TCA and recombinant TNFα significantly repressed AQP10 expression and NFκB p65/p50 directly bound to the AQP10 promoter and decreased its activity in PLC/RPF/5-*ASBT* cells and human obstructive cholestatic livers (Figure 7C–F). However, these alterations were abolished by BAY 11-7082, a specific inhibitor of NFκB signaling (Figure 7C). Taken together, our findings demonstrated that NFκB signaling activated by TNFα and TBA represses the expression of AQP10 in hepatocytes.

Conclusion

In summary, we first reported that hepatic AQP10 expression was significantly decreased in patients with obstructive cholestasis and revealed its functional role and regulatory mechanism in human obstructive cholestasis. The hepatocyte-specific overexpression of human AQP10 significantly attenuated cholestatic liver injury and intrahepatic BA accumulation in BDL mice. Therefore, overexpression of AQP10 in the liver may be a valuable strategy for cholestasis intervention.

Supplementary Materials

Material associated with this article can be found in the online version at https://doi.org/10.1016/j.gastha.2022.11.002.

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Jin Chai and Dequn Sun conceived and designed the experiments; Min Liao, Wenjing Yu, Qiaoling Xie, Liangjun Zhang, Ling Li, Ying Cheng, Qiong Pan, Nan Zhao, and Xiaoxun Zhang performed the experiments; Min Liao, Wenjing Yu, Qiong Pan, Liangjun Zhang, and Ying Chen analyzed the data; Ling Li and Nan Zhao contributed to reagents/materials/analysis tools; Min Liao ,Wenjing Yu, Liangjun Zhang, and Jin Chai wrote the article.

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The corresponding author, on behalf of all authors, jointly and severally, certifies that their institution has approved the protocol for any investigation involving humans or animals and that all experimentation was conducted in conformity with ethical and humane principles of research.

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Data, analytic methods, and study materials will not be made available to other researchers.

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