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IGFBP4 is a Metric for Primary Biliary Cholangitis and Attenuates Biliary Epithelial Cell Injury

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ABSTRACT

Background and Aims: Immune-mediated bile duct injury is the primary histological feature of autoimmune cholestatic liver diseases. Macrophages, the most abundant immune cell population in the liver, have been postulated to play a critical role in biliary repair. However, it is unclear whether activated macrophages interact with injured biliary epithelial cells.

Methods: We evaluated the expression of insulin-like growth factor-binding protein 4 (IGFBP4) in primary monocytes, MDMΦ, serum, and liver tissue sections from a total of 292 samples from PBC, PSC, and healthy controls using RNA-sequencing, ELISA, and immunohistochemistry analysis. The signal pathways involved in the effect of IGFBP4 in human intrahepatic biliary epithelial cells were examined by phospho-kinase arrays.

Results: Herein we demonstrate a role for insulin-like growth factor binding protein 4 (IGFBP4) in the interaction of macrophages and biliary cells. Importantly, the serum levels of IGFBP4 are significantly increased in PBC and negatively correlate with bilirubin levels. Furthermore, immunohistochemistry revealed an increase in IGFBP4 positive cells located not only in the periductal area but also around the portal tract in the PBC liver. In vitro study indicated that IGFBP4 protected biliary cells from bile salt-induced cell injury and promoted biliary cell proliferation, which was associated with reduced expression of the bile acid receptor TGR5, bile acid efflux transporter SLCO3A1, and activation of the GSK-3β/β-catenin signalling pathway.

Conclusions: These data highlight that IGFBP4 not only serves as a potential biomarker for PBC but also plays a protective role against bile duct injury.

Abbreviations: AMAs, anti-mitochondrial antibodies; BA, Bile acid; DB, direct bilirubin titre; ELISA, enzyme-linked immunoassay; GCDC, glycochenodeoxycholate; GM-CSF, granulocyte-macrophage colony-stimulating factor; HC, healthy controls; HiBECs, human intrahepatic biliary epithelial cells; IBD, inflammatory bowel disease; IGFBP, insulin-like growth factor binding protein; IGFBP4, insulin-like growth factor-binding protein 4; IGFs, insulin growth factors; IHC, immunohistochemical; LTB, lymphotoxin beta; MDMϕs, Monocyte-Derived Macrophages; MMP7, matrix metalloproteinase; MRP3, multidrug resistance-associated protein 3; MSCs, mesenchymal stem cells; NLRP3, NLR family pyrin domain containing 3; PBC, primary biliary cholangitis; PSC, primary sclerosing cholangitis; SLCO3A1, solute carrier organic anion transporter family member 3A1; TB, total bilirubin titre; TBS, Tris-buffered saline; TGR5, G-protein-coupled bile acid receptor Gpbar1; TRPV4, transient receptor potential cation channel subfamily V member 4.

Highlights

- Serum IGFBP4 levels are significantly elevated in patients with primary biliary cholangitis (PBC) and inversely correlate with bilirubin levels.
- IGPBP4-positive cells accumulate in periductal and portal tract areas of PBC liver sections.
- Macrophage-derived IGFBP4 mediates crosstalk between macrophages and injured biliary epithelial cells in PBC.
- IGFBP4 protects biliary epithelial cells from bile salt-induced injury and promotes cell proliferation in vitro. The protective effects involve the modulation of bile acid transporters and activation of the GSK-3β/β-catenin signalling pathway.

1 | Introduction

Autoimmune cholestatic liver diseases, which include primary biliary cholangitis (PBC) and primary sclerosing cholangitis (PSC), are characterised by immune-mediated progressive biliary destruction and impaired bile flow [1]. Specifically, PBC is immunologically distinguished by the presence of anti-mitochondrial antibodies (AMAs), infiltration of T cells around portal areas and progressive destruction of intrahepatic small bile ducts, which result in chronic cholestasis, inflammation, fibrosis and cirrhosis [1]. Conversely, PSC is characterised by fibrotic strictures, dilations of large intrahepatic and extrahepatic bile ducts, and a strong association with inflammatory bowel disease (IBD). Patients with PSC also have a significant risk of developing cholangiocarcinoma [1].

Accumulative data suggests the involvement of innate immunity and particularly macrophages in the pathogenesis of PBC [2, 3]. Our previous study demonstrated that activation of macrophages from patients with PBC, but not HC, in the presence of apoptotic bodies from HiBEC blebs and AMAs led to a significantly robust production of proinflammatory cytokines [3]. IGFBP4 is associated with M1 macrophages polarisation and activation [3–5]. An elevated IGFBP4 expression level has been reported in human monocyte to macrophage polarisation towards the M1 phenotype [6]. Importantly, IGFBP4 has been implicated to promote a pro-inflammatory response [7]. IGFBP4 is the smallest of the insulin-like growth factor binding protein (IGFBP) family with high affinity to insulin growth factors (IGFs), which exerts critical and diverse functions in normal development, growth, metabolism and homeostasis [8]. Overexpression of IGFBP4 has been reported in lupus nephritis [9], rheumatoid arthritis (RA), and T1DM [10]; serum IGFBP4 levels correlate with the severity of renal pathology in lupus and levels of IGFBP4 fragments positively associate with cardiovascular mortality rates in T1DM [9, 11]. It remains unclear whether and how IGFBP4 participates in the pathogenesis of autoimmune diseases.

In this study, we evaluated the expression levels of IGFBP4 in monocytes, macrophages, serum, and liver tissue sections from PBC, PSC and healthy controls. We further addressed its potential functional role under normal circumstances and in autoimmune cholestatic liver disease. Our data demonstrate that IGFBP4 expression is significantly elevated in activated MDMΦ, sera, and also elevated in the liver in PBC compared to patients with PSC and healthy controls (HC). Moreover, the data suggest that IGFBP4 has the potential to ameliorate biliary cells from bile salt injury and promote the proliferation of biliary cells through the *GSK-3β*/β-catenin signal pathway. Our findings highlight a potential protective role of MDMΦ via IGFBP4 on bile duct injury in PBC.

2 | Materials and Methods

2.1 | Study Subjects

A total of 258 human blood samples including 88 with PSC, 98 with PBC (90 AMA positive and 8 AMA negative PBC), and 72 healthy controls (HC) from 2008 to 2015 were collected from the University of California, Davis, and Humanitas Clinical and Research Center (Table 1). The HCs were selected and preferentially age and gender matched to PBC. The diagnosis of PSC and PBC was based on established international criteria [12, 13]. The sample size was calculated by Power/Sample Size Calculator based on our preliminary results of a small cohort study to achieve 80% power.

2.2 | Detection of IGFBP4 in Sera and Liver

Quantification of serum IGFBP4 was performed by Human IGFBP4 enzyme-linked immunoassay (ELISA) Kit (Thermo Fisher Scientific, EHIGFBP4). Evaluation of IGFBP4 expression in liver sections was conducted by immunohistochemical (IHC) staining. 3–5 μm sections were prepared from formalin-fixed paraffin-embedded liver tissue block and subjected to antigen retrieval with a Pascal Pressure Chamber (Agilent, Santa Clara, CA, US), antigen retrieval solution, and thereafter incubated with primary antibody for 1 h at room temperature. After washing with Tris-buffered saline (TBS), the sections were incubated with secondary antibodies (EnVision+ Single Reagent (HRP, Mouse), Agilent, Santa Clara, CA, US) for 1 h at room temperature. After washing, antibody binding was detected using 3,3′-Diaminobenzidine tetrahydrochloride (DAB) as a substrate for colour development. Antigenicity recovery by each retrieval solution was evaluated using a scoring system by a blinded pathologist. The degree of staining was scored by an unbias observer as follows: staining of the positive control specimen = 3, weaker than the control but positive = 2, slightly positive = 1, and no staining = 0 [14].

2.3 | Induction of Apoptosis and Apoptotic Bodies Isolation in Human Intrahepatic Biliary Epithelial Cells

Human intrahepatic biliary epithelial cells (HiBECs) from healthy donors, purchased from ScienCell (Carlsbad, CA). HiBECs were cultured in Epithelial Cell Medium (EpiCM,

TABLE 1 | General characteristics of study subjects.

	PSC	PBC		HC
Sample, *n*	88	98		72
AMA	—	Positive	Negative	—
n	—	90	8	—
Age (year), median (IQR)	42.5 (33.3–54.8)	58.5 (51.0–65.0)	64.5 (52.5–66.0)	55.0 (49.3–61.0)
Sex, female, *n*	45	83	8	56
Histological stage, *n*[a]	—	55	3	—
0	—	3	1	—
I	—	30	1	—
II	—	9	1	—
III	—	9	0	—
IV	—	4	0	—
Histological stage, *n*[a]	37	—	—	—
I—II	25	—	—	—
III–IV	12	—	—	—
Disease duration (month)[a]	79 (0–300)	64 (0–244)	79 (0–156)	—
Mayo PSC risk score[a]	0.10 (−2.03–2.85)	—		—
AST (IU/L)[a]	52 (10–212)	37 (10–149)	29 (13–48)	—
ALT (IU/L)[a]	67 (5–207)	43 (7–217)	33 (11–71)	—
ALP (IU/L)[a]	255 (49–817)	228 (41–1533)	149 (47–337)	—
TBil (mg/dL)[a]	1.36 (0.30–4.90)	0.76 (0.14–2.55)	0.60 (0.42–0.85)	—
DBil (mg/dL)[a]	0.67 (0.09–3.80)	0.29 (0.08–1.25)	0.21 (0.14–0.40)	—
Platelet (10^9/L)[a]	247 (105–440)	235 (25–459)	239 (164–350)	—
Albumin (mg/dL)[a]	3.84 (2.40–4.87)	—		—
Hb (mg/dL)[a]	13.26 (7.90–16.30)	—		—
UDCA, *n*[a]	65	72	6	—
Vitamin D, *n*[a]	19	35	4	—

Abbreviations: ALP, alkaline phosphatase; ALT, alanine aminotransferase; AMA, anti-mitochondrial antibody; AST, aspartate aminotransferase; DBil, direct bilirubin; Hb, haemoglobin; HC, health control; IBD, inflammatory bowel disease; IQR, interquartile range; PBC, primary biliary cholangitis; PSC, primary sclerosing cholangitis; TBil, total bilirubin; UDCA, ursodeoxycholic acid.
[a]Missing value exists.

ScienCell) supplemented with 2% fetal bovine serum, epithelial cell growth supplement (ScienCell), and 1% antibiotic solution (P/S) at 37°C in a humidified 5% CO2 incubator. To induce apoptosis, HiBECs were treated with sodium glycochenodeoxycholate (GCDC 200 nM) in the presence or absence of recombinant human IGFBP4 (500 ng/mL) (Biolegend) for 48 h [15]. After the induction of apoptosis, supernatants were collected and centrifuged twice (at 500 *g* for 5 min) for removal of intact cells. The supernatant fluid was then passed through a 1.2 um nonpyrogenic, hydrophilic syringe filter. After centrifugation at 100000 *g* for 45 min, the pellet containing apoptotic bodies was resuspended in RPMI medium. The suspension was immediately used for coculture with MDMϕs.

2.4 | Generation of Monocyte-Derived Macrophages (MDMϕs)

To generate MDMϕs, human mononuclear cells were isolated from peripheral blood using Histopaque-1077 (Sigma-Aldrich) density gradient centrifugation, and then subjected to anti-CD14 conjugated microbead by magnetic separation (Miltenyi Biotec) to obtain CD14+ monocytes. Aliquots of monocytes (1×10^6/mL) were then resuspended in Roswell Park Memorial Institute (RPMI) culture medium containing 10% heat inactivated fetal bovine serum (FBS) supplemented with 100 ng/mL granulocyte- macrophage colony-stimulating factor (GM-CSF). Cells were cultured for 5 days (fresh culture

medium was supplied at Day 3) in a humidified 5% CO2 incubator [3]. For M1 polarisation, MDMϕs from healthy controls and PBC were stimulated with 20 ng/mL interferon gamma (IFN-γ; ABclonal) and 50 ng/mL lipopolysaccharide (ABclonal) during the last 24 h of culture.

2.5 | Antibody Reagents and AMA Purification

Human immunoglobulin G (IgG) was purified from PBC sera using a protein G column (Pierce). Levels of AMAs were detected by ELISA [16]. Briefly, 96-well ELISA plates were coated with individual recombinant PDC-E2 protein (10 microgram/mL) in carbonate coating buffer at 4°C overnight, blocked with 3% non-fat dry milk in PBS, and incubated with a 1:2000 dilution of the serum samples for 1 h. The plates were then washed with PBS containing 0.05% Tween 20 and incubated for 1 h with a predetermined optimised dilution of horse-radish peroxidase (HRP) conjugated anti-human IgG (Invitrogen, Carlsbad, CA), and washed and developed with BD OptEIA Substrate (BD Biosciences, San Diego, CA). Positive and negative control serum samples were included throughout.

2.6 | Bulk RNA Sequencing

MDMϕs from 3 HC and 3 PBC patients were treated with AMA and apoptotic bodies from HiBECs as described [3]. RNA extraction, library preparation and sequencing were performed on the Illumina HiSeq2500 platform in a 2×150bp paired-end configuration in Rapid Run mode, with a total of at least 120 million reads per lane in GENEWIZ (South Plainfield, NJ).

2.7 | Cell Proliferation Assay

Cell proliferation was detected using an EdU assay. HiBECs were seeded (1×10^5/well) in a 12-well microtiter plate in a final volume of 500 μL/well culture medium in the absence or presence of recombinant IGFBP4 (500 ng/mL) for 14 h. Thereafter, EdU solution was added to each well and incubated for 2 h. After fixing, a click reaction was performed according to the manufacturer's protocol (C0078S, Beyotime, Beijing, China). DAPI solution was used to stain the nucleic acids. The fluorescence signals were measured under a fluorescence microscope and FACScan II, and the proportions of Edu positive (Edu+) cells were analysed by Image J software and FlowJo.

2.8 | Phospho-Kinase Arrays

To examine IGFBP4 activated signal pathways in HiBECs, we conducted Phospho-antibody array analysis using the Proteome Profiler Human Phospho-Kinase Array (R&D Systems). Briefly, HiBECs (3×10^5/well) were cultured in 6-well plate in Epithelial Cell Medium without FBS and treated with GCDC (200 nM) in the presence or absence of IGFBP4 (500 ng/mL) for 48 h. Cells were then harvested and solubilised at 1×10^7 cells/mL in cell lysis buffer (R&D Systems). Protein concentrations were determined using a BCA protein assay kit (Thermo Fisher Scientific). Array membranes were first blocked with Array Buffer 1 (R&D Systems) and then incubated with 600 μg of cell lysate overnight at 4°C. After washing with wash buffer, the membranes were incubated with biotinylated detection antibodies and followed by streptavidin-HRP. Chemiluminescent detection reagents (R&D Systems) were used to detect the spot densities. Array images were analysed using ImageJ software.

2.9 | Propensity Score Matching

The subjects were matched using the propensity score matching method proposed by Rubin and Rosenbaum [17]. The score was calculated with SPSS v.24.0 (IBM Corporation, Armonk, NY, USA). The propensity score for a subject was analysed given the covariates of total bilirubin and direct bilirubin using a logistic regression model. Each subject in the early-stage PSC group was matched to the individual with the closest propensity score in the early-stage PBC group. Both individuals were then eliminated from the next matching. The inclusion criteria for variables were a monotonized p value of < 0.2. Afterward, we adopted 1:1 nearest neighbour matching without substitution to make sure that interblock bias was minimal. According to a strict matching algorithm, the nearest neighbour matching matched each variable in the high group to a variable in the low group with the closest propensity score. The minimal propensity scores were matched for each participant. We tested many calliper widths. When the calliper width was 0.01, we got the optimal balance between homogeneity and reserved sample size.

2.10 | Statistical Analysis

All statistical analyses were performed using SPSS v.24.0 (IBM Corporation, Armonk, NY, USA) and GraphPad Prism software version 8.0.1 (GraphPad). The data were plotted using GraphPad. The ages of subjects were presented as medians with 25th and 75th quartiles (Q1, Q3). Continuous variables were otherwise expressed as mean values with standard error of the mean or means with ranges and compared using Student t-test as well as one-way ANOVA followed by Tukey's multiple comparisons test. The univariate correlation analyses of serum IGFBP4 and total bilirubin or direct bilirubin of PBC were determined using Spearman's rank coefficient (Spearman's rho). Sensitivity of the area under the curves (AUCs) was calculated with DeLong's method. Optimal cut-off was identified by the Youden index.

3 | Results

3.1 | Activated MDMϕs From PBC Produce an Increased Level of IGFBP4 Upon Exposure to Blebs and AMA

IGFBP4 is associated with M1 macrophages polarisation and activation. To examine whether activated PBC MDMϕs produce higher levels of IGFBP4 than HC MDMϕs, MDMϕs from

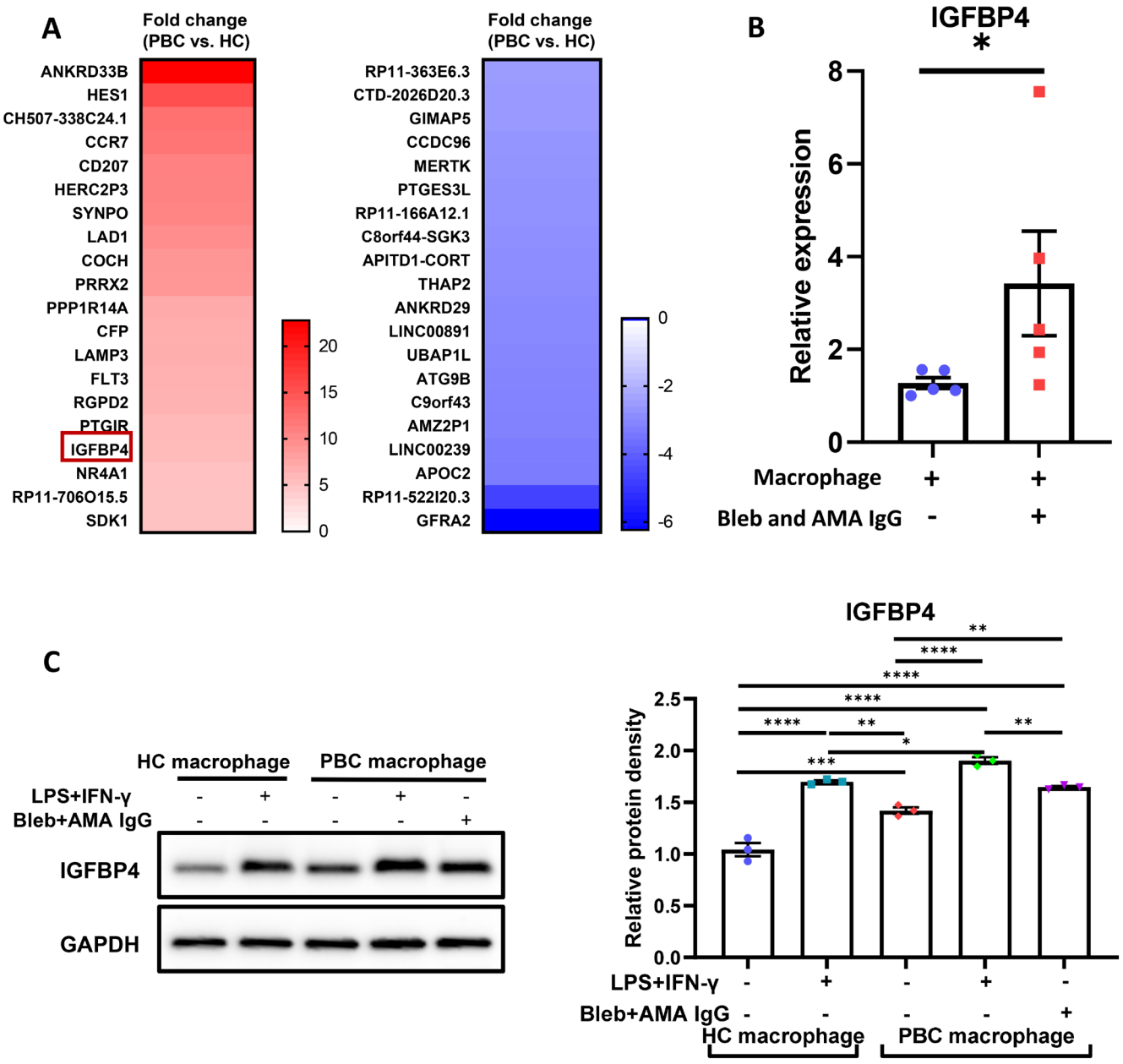

FIGURE 1 | The expression of IGFBP4 in activated monocyte-derived macrophages in PBC. (A) RNA sequence analysis of monocyte-derived macrophages, treated with anti-mitochondrial antibodies and biliary apotopes, from PBC ($n = 3$) and HC ($n = 3$). (B) RT-qPCR analysis of IGFBP4 in control monocyte-derived macrophages and activated monocyte-derived macrophages from PBC. (C) Western blot analysis of IGFBP4 in monocyte-derived macrophages from control and activated monocyte-derived macrophages from PBC. Data are presented as mean ± SEM. Statistical analysis was performed using Student T-test. Multiple categorical variables were compared using One-way ANOVA followed by Bonferroni multiple comparison test. *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$; ****$p < 0.0001$.

3 HC and 3 PBC patients were treated with HiBEC blebs and AMAs, and then subjected to RNA sequencing. The expression of IGFBP4 was significantly higher (7-fold higher) in PBC MDMϕs than HC MDMϕs (OR = 6.087, $p = 0.033$) (Figure 1A). To further assess whether the elevated expression level by PBC macrophages is due to the response to HiBEC blebs and AMAs, we compared IGFBP4 levels of PBC MDMϕs with and without treatment of HiBEC blebs and AMAs. PBC MDMϕs activated with HiBEC blebs and AMA produced increased expression of IGFBP4 as compared with untreated PBC MDMϕs, suggesting the IGFBP4 expression was triggered by HiBEC blebs and AMAs stimulation (Figure 1B,C).

3.2 | IGFBP4 Expression Is Significantly Elevated in Patients With PBC

Serum IGFBP4 levels were significantly elevated in PBC ($n = 96$) and PSC ($n = 69$) compared to HC ($n = 39$). There was a 7.2-fold increase in serum levels of IGFBP4 in PBC as compared to HC and a 3.9-fold increase in PSC (Figure 2A). To address whether an increase of IGFBP4 can also be evident in the liver, immunohistochemistry was performed to evaluate the expression of IGFBP4 in liver sections (Figure 2B). Although an increase of IGFBP4 was detected in the serum from PSC, only a few positive IGFBP4 cells were detected in two out of

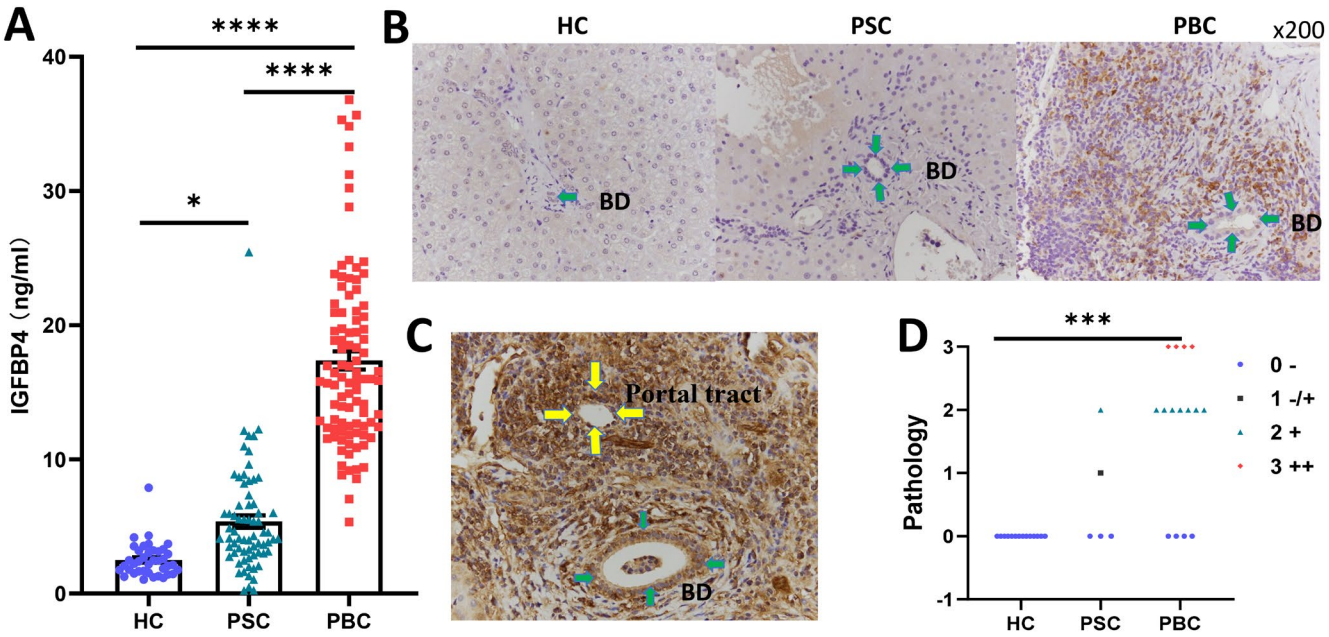

FIGURE 2 | The expression of IGFBP4 in serum and liver tissues in PBC, PSC and HC. (A) Quantification of serum IGFBP4 levels in PBC ($n = 96$), PSC ($n = 69$) and HC ($n = 39$). (B) Immunohistochemistry of IGFBP4 in liver of PBC, PSC and HC. (C) A representative image of IHC staining for IGFBP4 (high magnificent power field x400) (D) Immunohistochemistry scores of IGFBP4 in PBC, PSC and HC. Yellow arrows highlight the portal tract; Blue arrows highlight bile ducts (BD); Data are presented as mean ± SEM. Continuous variables were compared using one-way ANOVA followed by Bonferroni multiple comparison tests between PBC, PSC and HC. Categorical variables were compared with the Fisher's Exact Test between PBC, PSC and HC. *$p < 0.05$; ***$p < 0.001$; ****$p < 0.0001$.

five PSC liver sections. No IGFBP4 positive staining was detected in any of the normal livers (Figure 2D). In contrast, in the PBC liver, IGFBP4 positive stain cells were located not only in the periductal area but also found diffusely around the portal tract (Figure 2C). Altogether, our data indicated that IGFBP4 expression was significantly upregulated in circulating blood and liver of PBC.

3.3 | Serum IGFBP4 Level Inversely Correlates With Disease Stage and Bilirubin Titre

We examined whether the serum level of IGFBP4 correlated with characteristic clinical parameters of PBC. The level of serum IGFBP4 was significantly higher in early stage PBC patients than in end stage PBC patients (Figure 3A). Furthermore, the level of serum IGFBP4 was inversely correlated with direct bilirubin titre (DB) and total bilirubin titre (TB) in PBC (Figure 3C,D).

To further investigate the discriminative power of serum IGFBP4 levels for PBC, receiver operating characteristic (ROC) curves analysis was performed using a logistic regression model. Serum IGFBP4 displayed substantial discriminative power between PBC, PSC, and HC. The ROC curves based on serum IGFBP4 distinguished PBC from HC (AUC = 0.9995; $p = 1.1049E\text{-}19$; Figure 4A), and from PSC (AUC = 0.9657; $p = 2.1674E\text{-}24$; Figure 4B); additionally, the serum IGFBP4 level distinguished PSC from HC (AUC = 0.8019; $p = 2.0265E\text{-}7$; Figure 4C).

Antimitochondrial antibodies (AMAs) were detected in the sera of > 90% of PBC patients. Currently, the main challenge for diagnosis is distinguishing patients without detectable AMA levels from those with suspected PBC. In our result, serum IGFBP4 could distinguish AMA negative PBC from PSC and HC (Figure 4D). There was no significant difference in serum IGFBP4 between AMA positive PBC and AMA negative PBC (Figure 3B). Importantly, serum level of IGFBP4 could distinguish early-stage PBC from early-stage PSC after adjusting for bilirubin levels (Figure S2 and Table S1). The combination of AMA and IGFBP4 may contribute to improving the diagnosis of PBC.

3.4 | IGFBP4 Promotes the Proliferation of Biliary Cell and Rescues Biliary Cell From Bile Salt Induced Apoptosis

Given serum IGFBP4 levels were inversely correlated with bilirubin titre, we further addressed the function of IGFBP4 on cholangiocytes. As bile duct injury is commonly seen in PBC patients, partly due to the retention of bile acid and toxic substances, GCDC was utilised to induce biliary cell injury in vitro. The viability and proliferation of HiBECs in the absence and presence of IGFBP4 were measured using our EdU assay. Our data demonstrate that IGFBP4 promotes the proliferation of HiBECs (Figure 5A,B). Next, we explored whether IGFBP4 influences bile salt-induced biliary cell injury. We treated HiBECs with GCDC in the presence or absence of IGFBP4. The expression of genes that are relevant to bile duct physiology and inflammatory response such as NLR family pyrin domain containing 3 (NLRP3) [18], matrix metalloproteinase 7 (MMP7), multidrug resistance-associated protein 3 (MRP3), G-protein-coupled bile acid receptor Gpbar1 (TGR5), solute carrier organic anion transporter family member 3A1 (SLCO3A1),

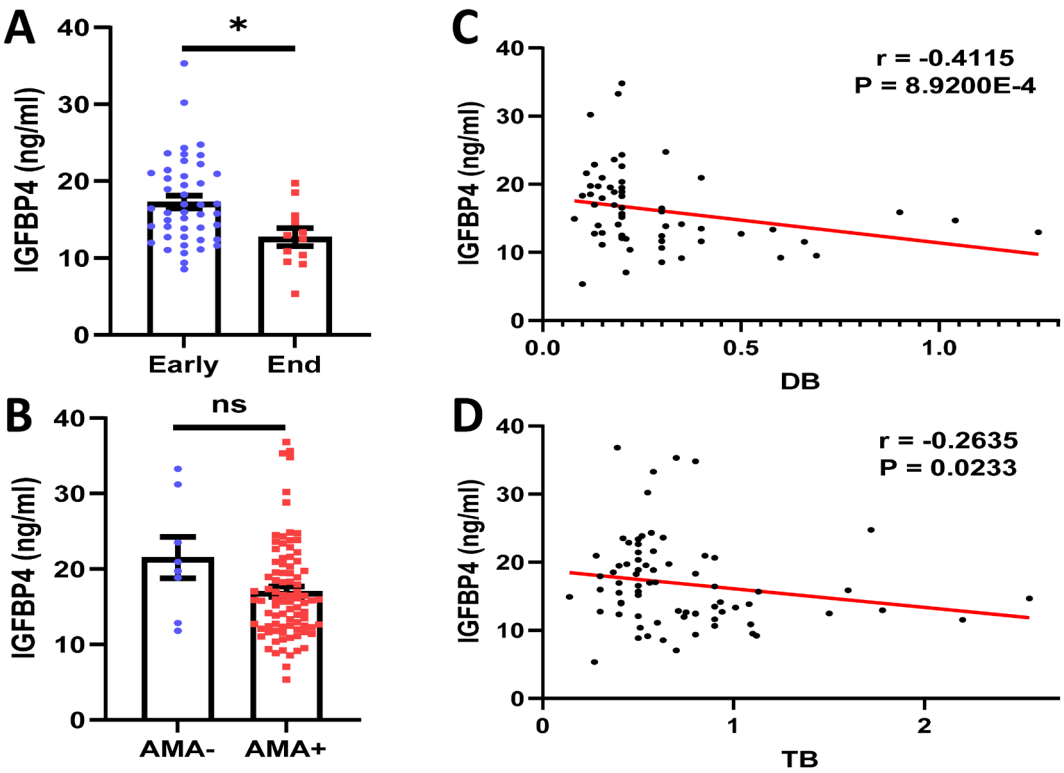

FIGURE 3 | Serum IGFBP4 was associated with pathology. (A) Comparison of serum IGFBP4 levels in early and end stage PBC. (B) Comparison of serum IGFBP4 levels in AMA negative and AMA positive PBC. (C) Correlation between serum IGFBP4 and DB of PBC. (D) Correlation between serum IGFBP4 and TB of PBC. Data are presented as mean ± SEM. Statistical analysis of continuous variable was performed using Student *t*-test. Statistical analysis of correlation was performed using Spearman correlations. $*p < 0.05$; Early, early stage; End, end stage.

transient receptor potential cation channel subfamily V member 4 (TRPV4) [19] and lymphotoxin beta (LTB) [20] were evaluated in treated HiBECs. As compared with GCDC-treated HiBECs, inflammasome NLRP3, the inducer of inflammatory response LTB, and the indicator of reactive bile ducts including TGR5, SLCO3A1, and TRPV4 were significantly decreased (Figure 5C) in GCDC-treated HiBECs in the presence of IGFBP4. Our data indicated IGFBP4 could potentially protect biliary cells from GCDC induced injury.

3.5 | Signalling Pathways Associated With the Protective Effect of IGFBP4 on Bile Salt Induced HiBEC Apoptosis

To explore the effect of downstream signalling of IGFBP4 on HiBECs in bile salt induced apoptosis, we analysed 11 differentially regulated signalling molecules in HiBECs using human phosphokinase microarray. HiBECs were exposed to GCDC (500 nM) for 48 h in the absence or presence of IGFBP4, and the phosphorylation levels of the individual kinases represented on the array were compared. IGFBP4 significantly increased the total protein level of β-catenin and phosphorylation levels of GSK-3β, Yes and Src, whereas IGFBP4 induced significant downregulation of *p38-α* phosphorylation in GCDC-treated HiBECs. Among these differentially expressed proteins, β-catenin was the most significantly regulated molecule upon IGFBP4 exposure in GCDC-treated HiBECs (Figure 6A,B). The expression of β-catenin, phosphorylation levels of β-catenin and GSK-3β were validated by Western blot (Figure 6C,D). Together, our results demonstrated that IGFBP4 promoted the expression of β-catenin in GCDC treated HiBECs, indicating IGFBP4 could prevent bile salt induced biliary cell injury through the activation of the *GSK-3β*/β-catenin and Src signalling pathways [21] (Figure 7 and Figure S1).

4 | Discussion

The specific destruction of small biliary cells in PBC is highly orchestrated via various biochemical and cellular interactions. Previous studies have demonstrated that AMA-exposed macrophages release excess proinflammatory cytokines in the presence of HiBEC apoptotic bodies [3, 15]. This observation provides a better understanding of the biliary specificity of PBC, the recurrence of the disease after liver transplantation, and possibly the basis for ursodiol in treating PBC [3].

In this study, we further investigated the molecular mechanisms of biliary injury and repair in PBC. First, our data from RNA sequencing of monocyte-derived macrophages from PBC and HC exposed to biliary apotopes and anti-mitochondrial antibodies clearly demonstrated that the expression of IGFBP4 is upregulated in PBC. In addition, our data demonstrate that serum levels of IGFBP4 are increased in PBC compared to PSC and HC. Further, IGFBP4 is markedly increased in liver in PBC compared to PSC or HC. These findings are consistent with studies of IGFBP4 in other autoimmune diseases, that is, type I diabetes [10, 11], RA [22], and SLE [9]. Immunohistochemistry analysis showed that IGFBP4 positive cells were primarily around the

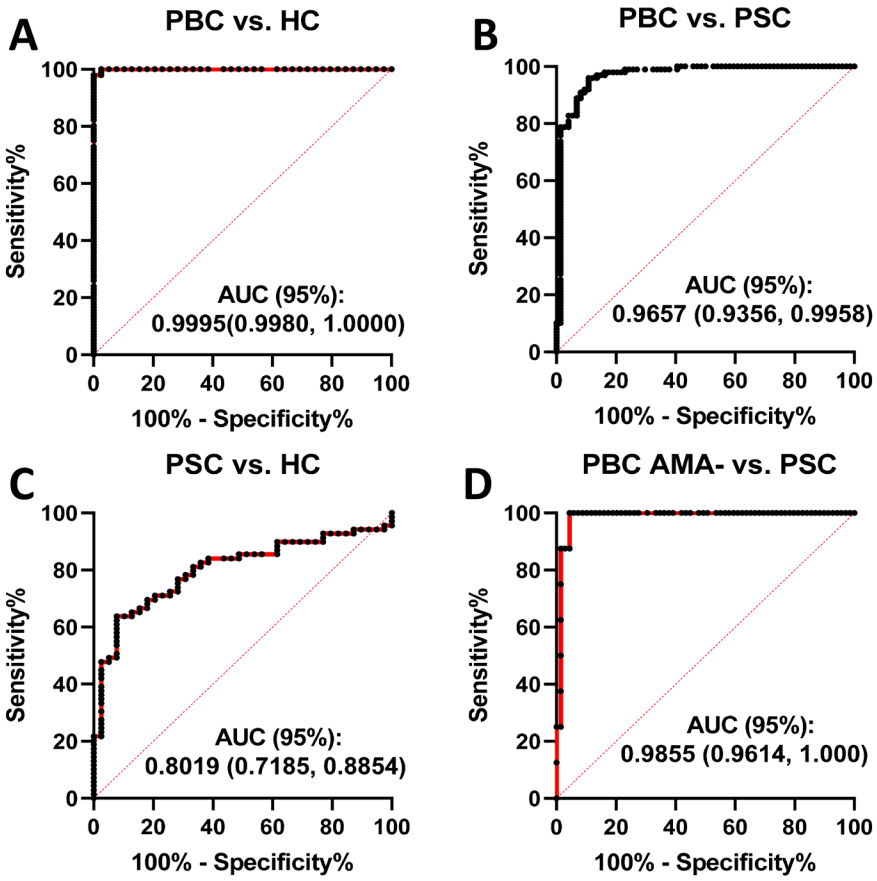

FIGURE 4 | ROC curve analysis illustrating the discriminative power of serum IGFBP4. Prediction of disease status between (A) PBC and HC, (B) PBC and PSC, (C) PSC and HC, and (D) AMA negative PBC and PSC. AUC, area under the curve; PBC AMA-, AMA negative PBC patients. AUC value is shown with a 95% confidence interval (CI).

bile ducts in PBC. IGFBP4 is a marker of M1 macrophages and is upregulated in monocyte polarisation towards M1 [23]. Such cellular changes can generate a proinflammatory environment around bile ducts and lead to biliary cell damage, which is a key feature in PBC.

Next, our data demonstrate that IGFBP4 promotes normal intrahepatic biliary cell proliferation. This result is consistent with several other studies focused on the function of IGFBP4 in cell growth. IGFBP4 is a cardiogenic growth factor, which enhances cardiomyocyte differentiation in the heart [24]. Additionally, IGFBP4 enhances angiogenesis and protects against ischemic diseases [25–27]. IGFBP4 promotes adipogenesis with sexual differences [28]. However, there are studies indicating that IGFBP4 inhibits cell growth. For example, IGFBP4 induced apoptosis and inhibited cell growth in young mesenchymal stem cells (MSCs) [29], HCC cells [30], and basal intestinal growths [31, 32]. The effect of IGFBP4 on cell proliferation is controversial, with data supporting both promoting and inhibiting cell growth. Together, the data suggest that IGFBP4 may have context dependent effects on cell growth. In particular, our results indicate that IGFBP4 promotes the proliferation of biliary cells in normal conditions.

The progressive destruction of intrahepatic small bile ducts is the primary pathological change in PBC. In this study, we utilised GCDC to induce biliary cell injury to mimic the destruction of intrahepatic small bile ducts. In our results, the component of inflammasome NLRP3 and the indicator of reactive bile ducts were significantly decreased after the treatment with IGFBP4. NLRP3 activation correlated with disease activity in PBC and contributed to liver injury in PSC [33, 34]. NLRP3 deletion led to decreased liver injury and inflammation in chronic cholestasis [33]. Therefore, the decreased expression of NLRP3 in GCDC treated biliary cells indicates that IGFBP4 has a protective role in bile duct injury. Bile acid (BA) metabolism related genes TGR5, SLCO3A1, TRPV4, and LTB were elevated in GCDC treated biliary cells. However, the expression of these BA metabolism related genes was downregulated when IGFBP4 was present. Previous studies demonstrated that TGR5, SLCO3A1, TRPV4, and LTB were increased in biliary cell under cholestasis [20, 35–38]. The pro-apoptosis effect of GCDC on biliary cell was reversed by IGFBP4, further suggesting IGFBP4 may attenuate bile salt induced biliary cell injury.

Our data also implies that the *GSK-3β*/β-catenin signalling pathway plays a role in IGFBP4 attenuation of bile salt-induced cholangiocyte injury. According to our data, IGFBP4 promotes the expression of β-catenin in bile salt-treated cholangiocytes. A similar result was also reported in human renal cell carcinoma; IGFBP4 activated the Wnt/β-catenin signalling pathway [39]. However, IGFBP4 has been reported to be an inhibitor of canonical Wnt signalling in cardiogenesis [24]. Recently, a study based on the Mdr2 KO mice model has

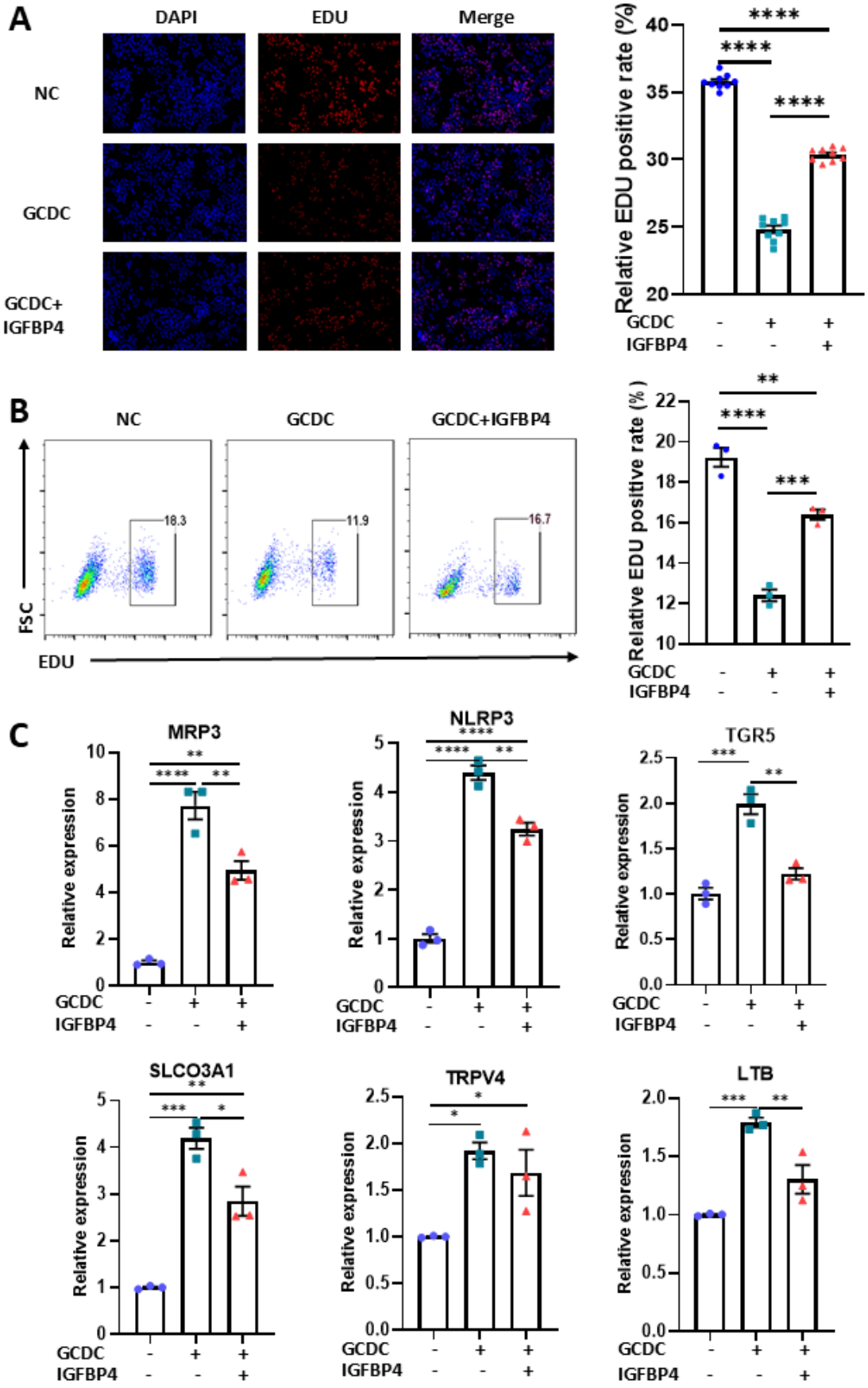

FIGURE 5 | Legend on next page.

FIGURE 5 | The effect of IGFBP4 on the proliferation of HiBECs. (A and B) Quantification of HiBECs proliferation (EdU assay). Control, HiBECs without treatment; IGFBP4, IGFBP4 treated HiBECs. (C) RT-qPCR analysis of gene expression in HiBECs treated with GCDC (200 nM) in the presence or absence of IGFBP4 (500 ng/mL) for 48 h. Data are presented as mean ± SEM. Statistical analysis of binary continuous variable was performed using Student *t*-test. Multiple categorical variables were compared using One-way ANOVA followed by Bonferroni multiple comparison test. *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$; ****$p < 0.0001$.

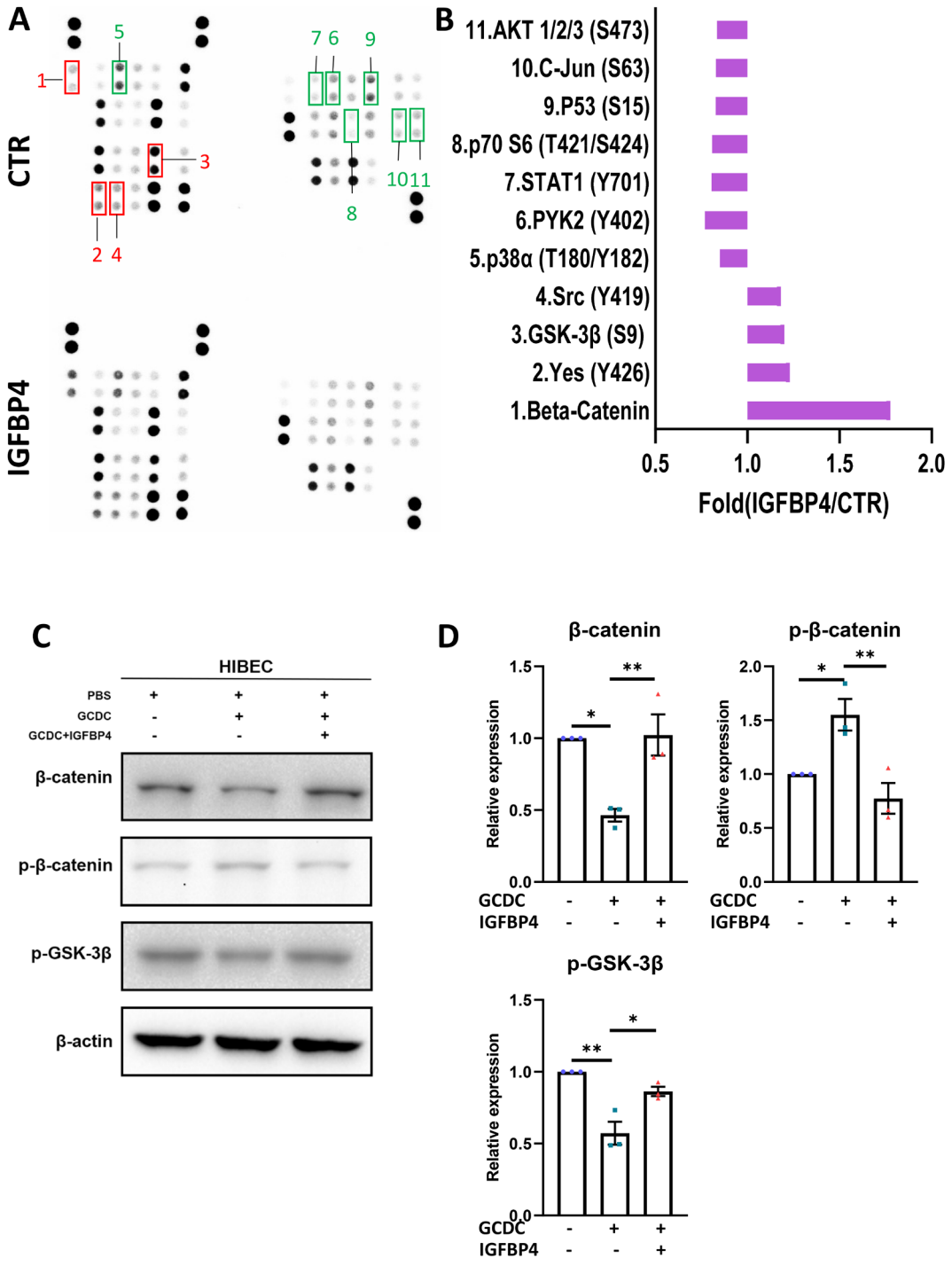

FIGURE 6 | Signalling pathways associated with IGFBP4 function on biliary cells. (A) Phospho-antibody array analysis of the signalling pathways. (B) Grey value analysis of phospho-antibody array. (C and D) Western blot analysis of expression of β-catenin, phosphorylation levels of β-catenin and GSK-3β in HIBECs treated with GCDC (200 nM) in the presence or absence of IGFBP4 (500 ng/mL) for 48 h. Data are presented as mean ± SEM. Statistical analysis of binary continuous variable was performed using the Student *t*-test. *$p < 0.05$; **$p < 0.01$.

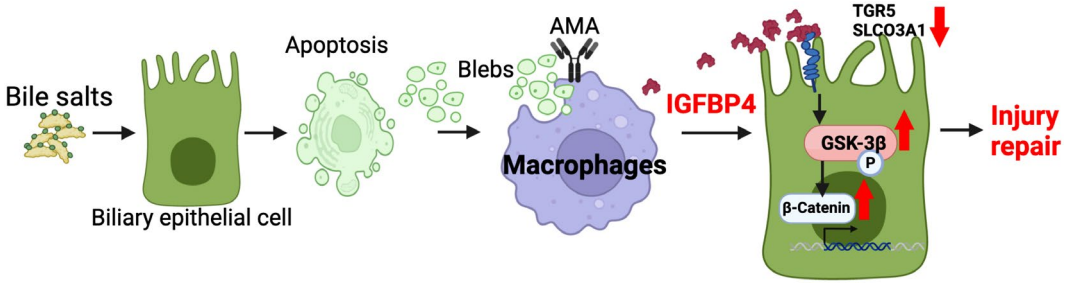

FIGURE 7 | Schematic illustration of IGFBP4 regulating *GSK-3β*/β-catenin signal pathway upon bile salt-induced biliary epithelial cell injury. IGFBP4 promotes the phosphorylation of the serine 9 residue of GSK3β (GSK3βS9), leading to GSK3β inactivation and stabilisation of β-catenin, allowing the latter to translocate into the nucleus and interact with transcription factors.

shown that inhibition of β-catenin exacerbates hepatobiliary injury. When β-catenin was knocked down in Mdr2 KO mice, parenchymal injury, ductular response, inflammation, fibrosis, oxidative stress and cholangiocyte senescence were increased, and BA synthesis, metabolism, and transporter genes were dysregulated [40]. Moreover, excess β-catenin transgenic mice exhibited an improvement in intrahepatic cholestasis, which indicates an improvement in hepatic repair [41]. This suggests that IGFBP4 may attenuate the bile salt induced injury of biliary cell through the *GSK-3β*/β-catenin signalling pathway.

Thus, it is worth noting that PBC patients have higher IGFBP4 expression levels than in PSC. The lower expression levels of IGFBP4 may contribute to the higher risk of hepatobiliary and colorectal cancers in PSC. In the advanced stage of PBC, IGFBP4 expression was decreased. As the fibrotic stage of the disease progressed, partial M1 macrophage reprogramming to M2 macrophages, which secrete various profibrotic factors such as TGF-β, PDGF and VEGF, activated myofibroblasts in the liver [42]. Therefore, the decrease of IGFBP4 could be partially due to increased M2 polarisation of macrophages in the end stage of PBC. Given the fact that HCC can occur in cirrhotic PBC patients, our data also suggest that IGFBP4 expression levels could serve as a risk indicator for the development of liver cancer; further studies are needed to assess this issue. A limitation of our study is the lack of in vivo experimental evidence from animal models to evaluate the relevance of IGFBP4 in the pathogenesis of PBC. Further studies are required to address the functional contribution of IGFBP4 in vivo.

In conclusion, our study identifies IGFBP4 as a novel potential biomarker for PBC and highlights the function and molecule pathways underlying IGFBP4 expression in human cholangiocytes.

Author Contributions

X.Z. participated in conducting experiments, acquiring data, analysing data, writing the manuscript; S.S., D.Y, Z.C. and T.T. participated in conducting experiments; P.S.C.L. and K.T. participated in revising the manuscript; P.I. and C.L.B. provided samples and participated in revising the manuscript; A.A.A. and W.M.R. participated in revising the manuscript; M.E.G. and W.Z. participated in designing research studies and writing the manuscript.

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Ethics Statement

The study was conducted under the Declaration of Helsinki and Istanbul. The study protocol was approved by the Institutional Review Board at the University of California, Davis and Humanitas Clinical and Research Center, Rozzano, Milan, Italy prior to the initiation of the study.

Consent

Informed consent was obtained from all patients.

Conflicts of Interest

The authors declare no conflicts of interest.

Data Availability Statement

The data that support the findings of this study are available on request from the corresponding author. The data are not publicly available due to privacy or ethical restrictions.

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Supporting Information

Additional supporting information can be found online in the Supporting Information section. **Figure S1:** The *GSK-3*β/β-catenin signalling pathways associated with IGFBP4 function on biliary cells. RT-qPCR analysis of *GSK-3*β/β-catenin signal pathway associated gene expression in HIBECs treated with GCDC (200 nM) in the presence or absence of IGFBP4 (500 ng/mL) for 48 h. Data are presented as mean ± SEM. Statistical analysis of binary continuous variable was performed using Student *t*-test. Multiple categorical variables were compared using One-way ANOVA followed by Bonferroni multiple comparison test. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$; ****, $p < 0.0001$. **Figure S2:** The discriminative power of serum level of IGFBP4. ROC curve analysis illustrating the discriminative performance of serum level of IGFBP4 for differentiating early-stage PBC from early-stage PSC following adjustment for bilirubin levels by propensity score matching. AUC, area under the curve; PBC AMA-, AMA negative PBC patients. The AUC is presented with a 95% confidence interval (CI). **Table S1:** General characteristics of early-stage PBC and early-stage PSC after adjusting for bilirubin levels by propensity score matching.