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Ischemic-Type Biliary Lesions (ITBLs) after Liver Transplantation is associated with High Expression of CYP3A5 rs776746 Allele A and the Immunilogical Therapeutic Mechanisms of Medicine

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Abstract

Ischemic-Type Biliary Lesions (ITBLs) are a common and difficult to treat biliary complication associated with liver transplantation. It is one of the main factors impacting long-term recipient and graft survival. It is therefore important and meaningful to investigate the mechanisms, effective prevention, and treatment of ITBLs. In this study, we retrospectively reviewed the records of 32 post-liver transplant patients with ITBLs, which were divided into those who received rapamycin and those who did not (controls). Using an All Prep DNA/RNA Mini Kit (Qiagen, Germany) according to the manufacturer's instructions, single nucleotide polymorphism (SNP) of cytochrome P450, family 3, subfamily A5 (CYP3A5) rs776746 were genotyped in both donors and recipients. There are 15 cases have one alleles A, 12 cases two alleles A and 5 cases three alleles A in both donors and recipients. With increasing numbers of CYP3A5 rs776746 alleles A, patients were found to have increasingly higher bile duct injury score at time points when ITBLs were identified. After rapamycin treatment, there was a significant improvement in liver function indexes, and the bile duct immunopathological damage alleviated significantly. These findings demonstrate that (i) Ischemic-Type Biliary Lesions (ITBLs) after liver transplantation is associated with high expression of CYP3A5 rs776746 allele A; and (ii) Rapamycin can stimulating FoxP3+ Treg cells, suppressing immunopathological damage, and promoting epithelial repair in bile ducts.

Keywords: CYP3A5; Ischemic-Type Biliary Lesions (ITBL); FoxP3+ Treg cells; Immunopathological damage

Introduction

Ischemic-type biliary lesion (ITBL) is one of the common and formidable complications after liver transplantation. It is the main cause leading to graft liver dysfunction and patients' death. In recent years, due to an increasing shortage of cadaveric donors, there has been an increase in the use of donation after cardiac death (DCD) liver graft, thus increasing the incidence of ITBLs. It is therefore important and meaningful to investigate the mechanisms, effective prevention, and treatment of ITBLs. CYP3A enzymes are the major metabolic enzymes of tacrolimus.

The rs776746 polymorphisms in intron 3 of CYP3A5 have been correlated with altered gene expression due to a splicing defect. These CYP3A5 rs776746 A genotype carriers associated with fast tacrolimus metabolism and lower C/D ratio. Based on our clinical experience, CYP3A5 rs776746 alleles A patients have higher bile duct injury, and rapamycin can not only improve liver function indexes in patients with ITBLs, but can also delay or suppress the occurrence of ITBLs and promote epithelial repair in the bile duct as indicated by histological studies, thereby preventing secondary liver transplantation. The present study aimed to investigate the pathogenic mechanisms of ITBLs, and the therapeutic mechanisms of rapamycin treatment for ITBLs.

Materials and Methods

Patients

This study was approved by the Institutional Review Board of our center, and because of its retrospective nature the requirement of informed patient consent was waived.

This retrospective study included patients with a confirmed diagnosis of ITBLs who received liver transplantation at our center from 2004 to 2010. The characteristics of the subjects in our study are detailed in Table 1. The diagnostic criteria for ITBLs were: 1) postoperative jaundice, usually occurred 3-6 months after transplantation; 2) destruction of non-anastomotic parts of the biliary tree confirmed by MRCP, ERCP, cholangiography, and other imaging studies, including segmental stenosis and expansion of bile ducts, filling defects, biliary sludge, biliary casts, or bile duct damage; 3) graft rejection, drug toxicity, and the recurrence of primary disease were ruled out by liver puncture biopsy; and 4) biliary casts removed by surgery or endoscopy (Figures 1 and 2).

Inclusion criteria for this study were: 1) ITBL was confirmed by clinical manifestations, imaging studies, and liver biopsy; 2) liver puncture biopsy was conducted before and after the ITBL was diagnosed; 3) a sufficient amount of biopsy specimen was obtained with no less than five portal areas; 4) good patient compliance and complete and reliable clinical follow-up data.

Patient ID	Sex	Age	Diagnose	Operation time	Warm ischemia (min)	Cool ischemia (min)	ITBL time after operation (months)	Clinical prognosis	Group
1	Male	52	HCC	2004-11-18	2	590	6	Cure	Control
2	Male	40	A on C	2004-11-26	5	710	8	Regraft	Control
3	Male	25	A on C	2004-12-21	4	850	10	Regraft	Therapy
4	Male	42	HCC	2004-12-24	4	740	10	Death	Control
5	Female	45	LC	2004-12-24	6	660	9	Regraft	Control
6	Female	43	HCC	2005-1-12	4	660	10	Death	Control
7	Male	61	HCC	2005-1-12	6	710	6	Survival	Control
8	Male	40	HCC	2005-1-19	5	585	7	Regraft	Control
9	Male	35	A on C	2005-2-2	4	685	10	Regraft	Control
10	Male	51	HCC	2005-4-1	2	468	6	Cure	Therapy
11	Female	43	PBC	2005-6-30	4	620	7	Death	Control
12	Male	42	A on C	2005-7-28	5	595	6	Regraft	Control
13	Male	31	LC	2005-8-10	4	655	10	Cure	Therapy
14	Male	28	A on C	2005-9-15	3	710	13	Cure	Therapy
15	Male	45	HCC	2005-9-23	6	540	7	Regraft	Therapy
16	Male	46	HCC	2005-10-19	3	630	9	Regraft	Control
17	Male	46	A on C	2006-1-13	3	920	5	Cure	Therapy
18	Male	46	A on C	2006-3-7	3.5	844	5	Cure	Therapy
19	Male	57	ALC	2006-3-12	1	690	5	Regraft	Therapy
20	Male	33	A on C	2006-3-17	2	872	6	Cure	Control
21	Male	41	HCC	2006-6-3	1.5	1120	10	Survival	Control
22	Female	52	PBC	2006-8-4	6	845	5	Cure	Therapy
23	Female	53	PBC	2006-11-17	1	540	7	Death	Therapy
24	Male	46	LC	2006-12-26	6	870	7	Cure	Therapy
25	Male	51	A on C	2007-2-9	1	780	7	Cure	Therapy
26	Female	19	ASH	2007-3-6	2	600	7	Regraft	Therapy
27	Male	51	LC	2007-11-30	3	878	5	Cure	Therapy
28	Female	50	A on C	2008-1-8	2	697	5	Regraft	Therapy
29	Male	42	HCC	2008-3-14	5	440	7	Cure	Therapy
30	Male	50	LC	2008-6-13	7	281	6	Survival	Therapy
31	Male	46	HCC	2008-7-6	3	420	5	Cure	Therapy
32	Male	49	LC	2008-12-5	6	480	5	Survival	Therapy

Table 1: Characteristics of the study patient cohort.

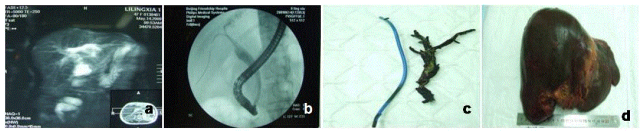

Figure 1: A 47-year-old female with ITBLs 7 months after liver transplantation. (a and b) Serious intrahepatic biliary damage was confirmed by MRCP and ERCP. (c) The biliary tree was removed during surgical exploration. (d) Severe cholestatic changes were seen in liver resection specimen following re-transplantation

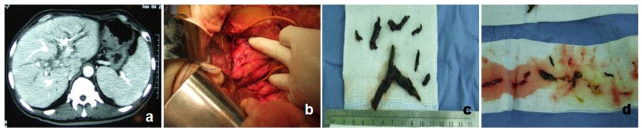

Figure 2: A 50-year-old male with ITBLs 6 months after liver transplantation. (a) MRI showed intrahepatic segmental stenosis and expansion and filling defect. (b-d) A number of biliary casts and biliary sludge were removed during surgical exploration

Exclusion criteria for this study was: 1) liver puncture biopsy was not conducted before or after the ITBL was diagnosed; 2) the amount of biopsy specimen was insufficient and less than five portal areas; 3) poor patient compliance and incomplete and truthless clinical follow-up data.

Based on the above inclusion criteria, a total of 32 patients were enrolled in the study, including 25 males (78.13%) and 7 females (21.87%) with a median age of 46 years (range, 19 - 61 years). The primary diseases included hepatocellular carcinoma in 11 patients, acute liver failure on chronic liver disease in 10 patients, decompensate liver cirrhosis secondary to hepatitis in 6 patients, primary biliary cirrhosis in 3 patients, severe subacute hepatitis in 1 patient, and alcoholic cirrhosis in 1 patient. Preoperative liver function Child class was grade A in 2 patients, grade B in 10 patients, and grade C in 20 patients.

All patients received deceased liver transplantation, including orthotopic liver transplantation in 10 patients and piggyback orthotopic liver transplantation in 22 patients. The procedure of biliary reconstruction included common bile duct to jejunum (CDJ) Roux-en-Y anastomosis in 1 patient, and common bile duct end-to-end anastomosis (CDC) in 31 patients. A good blood supply was maintained during anastomosis. T-tube drainage was placed for 11 patient-times, and withdrawn after 6-8 months following a conventional T-tube cholangiography. Mean donor warm ischemia time was 3.8 min (range, 1-7 min) and mean donor cold ischemic time was 678 min (range, 281-1,120 min). All patients received postoperative immunosuppressive therapy with tacrolimus (FK506), mycophenolate mofetil, and prednisolone. The time to onset of ITBL was 7.2 months (range, 5-13 months) after surgery. The biliary cast was removed by conventional bile duct exploration or endoscopy in 14 patients.

Drugs and reagents

Drugs and reagents included rapamycin (Wyeth Pharmaceuticals), anti-interleukin (IL)-2 monoclonal antibody (MA1-90700, Thermo), FoxP3 monoclonal antibody (13-4776-80, eBioscience), anti-IL-10 monoclonal antibody (MA1-82664, Thermoof), immunohistochemistry (IHC) Biotin Block Kit (BLK-0002, Maixia-Bio, China), DAB Detection Kit (DAB-0031, Maixia-Bio, China), HRP-Polymer anti-Mouse IHC Kit (SP-9000, Beijing Zhongshan Golden Bridge, Ltd.)

Treatment

All 32 patients were assigned selectively into the rapamycin group (19 cases) and a control group (13 cases) according to if they were treated by rapamycin or not. After the diagnosis of ITBL was confirmed, patients in the rapamycin group received a standard dose of rapamycin to maintain a blood concentration of 4-8 ng/ml, combined with immunosuppressive therapy consisting of tacrolimus, mycophenolate mofetil, and prednisolone. Patients in the control group received an immunosuppressive therapy consisting of tacrolimus, mycophenolate mofetil, and prednisolone. Drug dosage in each patient was adjusted according to blood concentration, liver function indexes, and clinical symptoms.

Clinical observations

Liver function tests were performed regularly using fasting blood samples. Serum transaminase (ALT and AST), bilirubin (TBil), bile duct enzymes (γ-GT, ALP), total bile acid (TBA), albumin (ALB), prealbumin (PALB), and cholinesterase (CHE) were detected by an automatic biochemical analyzer.

Clinical prognosis was determined by using Life Tables to calculate 1-year and 3-year graft survival rates (death or retransplantation were considered as graft loss).

Morphological changes in liver tissue were observed under a light microscope. Lesions in the hepatic portal bile duct included destruction and hyperplasia of the bile duct, and inflammatory cell infiltration in the portal area. Lesions were scored as no lesion (0), mild or local lesion (1 point), moderate lesion (2 points), severe lesion (3 points).

Detection of IL-2, FoxP3, and IL-10 expressions in the hepatic portal area

Immunohistochemistry staining was conducted following the standard procedures of deparaffinization, rehydration, antigen retrieval, dehydration and drying, and staining. The expression of IL-2, FoxP3 and IL-10 in the portal area was observed under a light microscope. IL-2 and IL-10 positive expressions were located in the cytoplasm, and the FoxP3 positive expression was located in the nucleus. A typical area was selected from each slice, and images were obtained at a magnification of 40. Ten consecutive visual fields were selected manually. Using Imagepro Plus 6.0 analysis software, the expressions of IL-2, Foxp3, and IL-10 in the sections were quantified. The positive staining area (area), the average optical density (OD), and the integral OD (IOD) of each visual field were measured and analyzed. The average optical density values were used for data analysis.

Extracting genomic DNA and genotyping

Using an All Prep DNA/RNA Mini Kit (Qiagen, Germany) according to the manufacturer's instructions, genomic DNA was isolated from both donor and recipient liver tissue, which were previously stored at -80ºC. Genotyping of SNPs was conducted by the Sequenom Mass ARRAY SNP genotyping platform (Sequenom, USA) [1]. The protocols included DNA and primer preparation, PCR amplification, SAP treatment, primer extension, resin cleanup, spotting primer extension products on Spectro-CHIP, and detection primer extension products by mass spectrometer.

Statistical analysis

SPSS 14.0 software was used for statistical analysis. Quantitative data were expressed as mean ± standard deviation. Student t-test and one-way ANOVA were used for the comparison between groups, respectively. A value of $P<0.05$ was considered to indicate statistical significance.

Results

Gene polymorphisms

In donor, 75% (24/32) was CYP3A5, rs776746 allele A carriers, and 78% (25/32) in recipients. There were no differences in allele A frequencies between donors and recipients. Genotype frequencies are shown in Table 2.

Genotypes	Frequency of donors	Frequency of recipients
AA	1	4
GA	23	21
GG	8	7

Table 2: Genotype frequencies of CYP3A5 rs776746.

Both donor and recipient CYP3A5 rs776746 allele A were all associated with fast tacrolimus metabolism. Therefore, we investigated alleles A of both donors and recipients in a combination analysis. The associations between the number of alleles A and bile duct injury score at the time ITBLs are shown in Figure 3. With increasing numbers of alleles A, patients were found to have increasingly higher bile duct injury score at the time ITBLs were diagnosed ($P<0.05$).

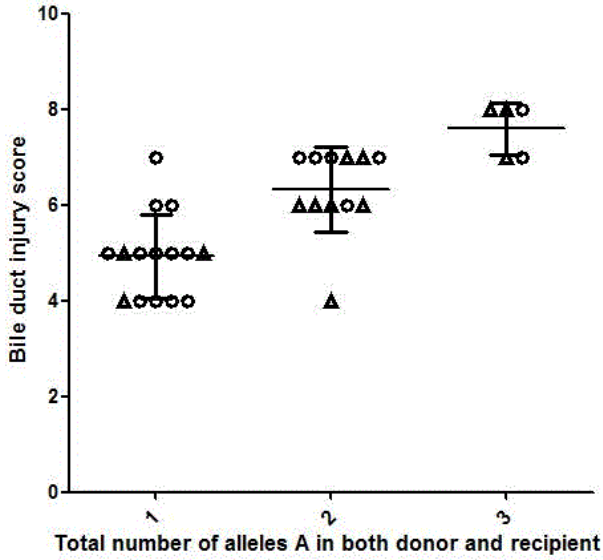

Figure 3: Association of donor and recipient CYP3A5 rs776746 allele A with bile duct injury score in post-liver transplantation patients with ITBLs. Data are shown as mean (±95%CI) (○: participants receiving rapamycin; Δ participants no receiving rapamycin)

Effects of rapamycin on liver function

As shown in Table 3, compared with pre-ITBL levels, there was a significant increase in serum levels of ALT, AST, Tbil, gamma-GT, and ALP and a significant decrease in the serum levels of PALB and CHE in both groups at the time of ITBL diagnosis (all, $P<0.05$). After 6 months treatment, no change was found in the serum levels of ALT, AST, Tbil, γ-GT, ALP, PALB, CHE, and ALB in the control group. However, the serum levels of ALT, AST, TBIL, gamma-GT, and ALP in the rapamycin group were significantly decreased compared with pre-treatment levels (all, $P<0.01$) and the post-treatment levels in the control group (all, $P<0.05$), while the serum levels of ALB, PALB, and CHE were significantly increased compared with pre-treatment levels (all $P<0.05$).

Marker of Liver Function	Group	No.	Pre-ITBL	ITBL	Post-therapy
ALT	Control	13	73.65 ± 29.11	290.21 ± 182.86$^{\$\$}$	294.37 ± 168.20
	Rapamycin	19	68.63 ± 30.17	336.03 ± 101.03$^{\&\&\&}$	146.21 ± 96.51$^{***\#}$
AST	Control	13	78.89 ± 26.03	350.4 ± 128.74$^{\$\$\$}$	272.65 ± 90.32
	Rapamycin	19	72.43 ± 34.01	351.9 ± 152.60$^{\&\&\&}$	153.21 ± 87.94***
TBil	Control	13	25.75 ± 7.94	135.22 ± 73.89$^{\$\$\$}$	143.27 ± 74.54
	Rapamycin	19	21.23 ± 5.76	111.4 ± 50.85$^{\&\&\&}$	43.05 ± 54.96$^{***\#\#\#}$
γ-GT	Control	13	127.52 ± 73.52	336.01 ± 144.05$^{\$\$}$	303.17 ± 160.59

	Rapamycin	19	118.16 ± 74.07	311.03 ± 199.52$^{\&\&}$	148.24 ± 71.48$^{**\#}$
ALP	Control	13	133.68 ± 82.28	387.1 ± 246.40$^{\$\$}$	451.77 ± 277.72
	Rapamycin	19	121.92 ± 82.15	336.96 ± 245.09$^{\&\&\&}$	151.62 ± 82.15$^{**\#\#\#}$
ALB	Control	13	37.4 ± 4.11	34.48 ± 4.91	35.25 ± 5.02
	Rapamycin	19	36.13 ± 6.47	33.13 ± 3.18	38.03 ± 5.32*
PALB	Control	13	222.48 ± 65.12	171.79 ± 44.30$^{\$}$	196.38 ± 53.17
	Rapamycin	19	215.38 ± 71.13	159.6 ± 65.03$^{\&\&}$	224.88 ± 50.21**
CHE	Control	13	4935.85 ± 1219.09	3883 ± 1031.19$^{\$}$	4140 ± 1289.20
	Rapamycin	19	4855.95 ± 1233.25	3981.32 ± 1069.74$^{\&}$	4894.47 ± 1345.48*

Table 3: Effects of rapamycin on liver function indexes in ITBL patients.

Data are presented as mean ± standard deviation.

$^{\$}$, $^{\$\$}$, $^{\$\$\$}$Compared with pre-ITBL levels in the control group, P<0.05, P<0.01, P<0.05 respectively.

$^{\&}$, $^{\&\&}$, $^{\&\&\&}$Compared with pre-ITBL levels in the rapamycin group, P<0.05, P<0.01, P<0.05 respectively.

*, **, ***Compared with ITBL levels in the rapamycin group, P<0.05, P<0.01, P<0.05 respectively.

$^{\#}$, $^{\#\#}$, $^{\#\#\#}$Compared with post-therapy levels in the control group, P<0.05, P<0.01, P<0.05 respectively.

Effects of rapamycin on prognosis

In the control group, there were 6 cases (46.15%) of liver retransplantation of which 1 patient died, 3 (23.08%) other deaths, 2 complete remissions (15.38%) with normal liver function indexes and histological examination results, and 2 (15.79%) stable cases. In the rapamycin group, there were 5 cases (26.32%) of liver retransplantation, 1 (5.26%) death, 10 (52.63%) complete remissions, and 3 (15.79%) stable cases. The 1-year and 3-year graft survival rates were 46.15% and 38.46%, respectively, in the control group and 89.47% and 77.54%, respectively, in the rapamycin group, which were significantly different between the 2 groups (P=0.007, P=0.020, respectively) (Table 4).

Group	No.	Graft Survival Rate (%)	
		1-year	3-year
Control	13	46.15 ± 7.19	38.46 ± 10.12
Rapamycin	19	89.47 ± 7.04**	77.54 ± 9.95*
Comparison between groups Pearson Chi-square		χ2=7.166 P=0.007	χ2=5.398 P=0.020

Table 4: Effects of rapamycin on the graft survival rate of ITBL patients

*Compared with the control group at the same stage, P<0.05

**Compared with the control group at the same stage, P<0.01

Effects of rapamycin on bile duct injury

Morphological changes in liver tissue were observed on hematoxylin and eosin (H&E) stained slides. As shown in Table 5, Figures 4 and 5, at the time of ITBL diagnosis the bile duct injury scores were significantly higher than pre-ITBL scores in both groups (all, P<0.001). No difference was found between the 2 groups before and after ITBLs occurred. After 6 months of treatment, there was no change in the bile duct injury score in the control group. However, the post-therapy score in the rapamycin group was significantly lower compared with previous scores (P<0.01), and the scores in the control group at the same time points (P<0.05).

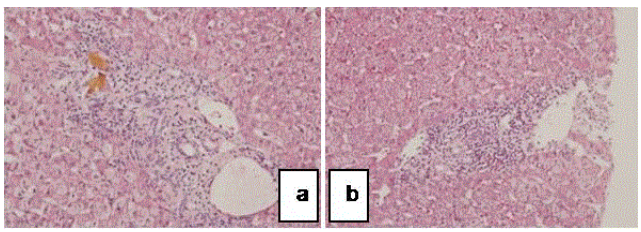

Figure 4: A 42-year-old male. ITBLs occurred 7 months after liver transplantation. a) Histopathological examination revealed portal area expansion, infiltration of a large number of mixed inflammatory cells, hyperplasia of the small bile ducts, partial epithelial vacuolation in the bile ducts, and lymphocytic infiltration in the bile ducts. Score = 7. b) After 6 months of rapamycin treatment. Histopathological examination revealed mild degeneration of the small bile ducts in the portal area, infiltration of a small amount of mixed inflammatory cells, occasional epithelial vacuolation in the bile ducts, mild fibrosis. Score = 4. (H&E ×400)

Group	No	Pre-ITBL	ITBL	Post-therapy
Control	13	2.77 ± 0.93	6.31 ± 0.85$^{\$\$\$}$	6.08 ± 1.75
Rapamycin	19	2.47 ± 0.77	6.11 ± 0.64$^{\&\&\&}$	4.42 ± 2.40$^{**\#}$

Table 5: Effects of rapamycin on pathological lesions of the bile duct in post-liver transplantation patients with ITBLs

Data are presented as mean ± standard deviation

$^{\$\$\$}$ P<0.001 compared with pre-ITBL score in the control group

$^{\&\&\&}$P<0.001compared with pre-ITBL score in the rapamycin group

**P<0.01compared with ITBL score in the rapamycin group

$^{\#}$P<0.05 compared with post-therapy score in the control group

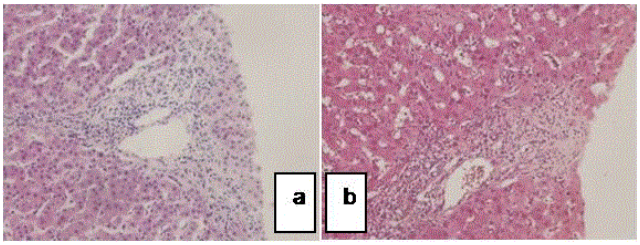

Figure 5: A 50-year-old female. a) ITBLs occurred 5 months after liver transplantation. Histopathological examination revealed portal area expansion, infiltration of a small to medium number of mixed inflammatory cells, a large amount of hyperplasia in small bile ducts, partial epithelial vacuolation in the bile duct, and occasional lymphocytic infiltration in the bile duct. Score = 6. b) After 6 months of rapamycin treatment. Histopathological examination revealed mild degeneration of the small bile ducts in the portal area, infiltration of a small number of mixed inflammatory cells, occasional epithelial vacuolation in the bile duct, and mild fibrosis. Score = 3. (H&E ×400)

Effects of rapamycin on IL-2, FoxP3 and IL-10 expressions in the portal area

As shown in Table 6, there was a significant increase in the OD values of IL-2 (P<0.01), but not in FoxP3 and IL-10 OD values at the time of ITBL diagnosis compared with pre-ITBL OD values in both groups. After 6 months of treatment, compared with their values at the time of ITBL diagnosis, there was no significant change in post-therapy OD values of IL-2, FoxP3, and IL-10 in the control group. However, there was a significant decrease in IL-2 OD value and a significant increase in FoxP3 and IL-10 OD values in the rapamycin group (P<0.05). The differences between 2 groups were significant (P<0.05).

Discussion

Ischemic-type biliary lesions (ITBLs) are a common biliary complication associated with liver transplantation, and are one of the main factors impacting long-term recipient and graft survival. In recent years, due to an increasing shortage of cadaveric donor, there is a gradual increasing application of the donation after cardiac death (DCD) liver graft. The incidence of ITBL is therefore increased [2-8], which becomes an important constraint for clinical efficacy of liver transplantation. The etiology and pathogenesis of ITBLs are not clear. At present, well-recognized pathogenic factors include prolonged donor cold and warm ischemic times, graft ischemia-reperfusion injury, ischemic injury of the bile duct due to postoperative blood supply insufficiency, autoimmune bile duct injury, drug-induced bile duct damage, and infection-induced bile duct damage [9]. In recent years, the incidence of post-transplant ITBLs has been dramatically reduced by strategies such as protecting the blood supply to the bile duct, using low viscosity perfusion solution to perfuse blood vessels and the bile duct, shortening the liver warm and cold ischemia times as much as possible, extracorporeal membrane oxygenation (ECMO) perfusion for DCD donor [10,11], and the prevention and rapid management of postoperative rejection and infections. However, the incidence of ITBLs is still as high as 5.3-19%, depending on the donor source.

Biliary epithelial cells are one of the most sensitive cells in the body to ischemia, hypoxia, and inflammatory injury. They contain a large number of class II histocompatibility antigens (MHC-II), and are thus a primary target of immunopathological damage after liver transplantation. In addition, biliary epithelial cell ischemic injury can first trigger a non-specific inflammatory reaction followed by an immunopathological reaction. Both pathological reactions can cause a cytokine cascade, resulting in a large amount of cytokine release. In addition, there are a large number of cytokine receptors on biliary epithelial cells, which play a role in lymphocyte chemotaxis and activation, thereby attracting intrahepatic lymphocytes and becoming the target of T-lymphocytes. The expression of MHC-II antigens in biliary epithelium increase after ischemic injury, which can then activate or aggravate immunopathological damage with ITBLs, thus becoming an important factor for persistent bile duct damage. Therefore, it can be inferred that suppression of immunopathological damage and inhibition of the expression or release of various cytokines may be able to reduce the extent of bile duct injury post-transplantion.

Group	No	IL-2			FoxP3			IL-10		
		Pre-ITBL	ITBL	Post-therapy	Pre-ITBL	ITBL	Post-therapy	Pre-ITBL	ITBL	Post-therapy
Control	13	0.0950 ± 0.0243	0.1385 ± 0.0500$^{\$\$}$	0.1276 ± 0.0295	0.1701 ± 0.0460	0.1433 ± 0.0469	0.1465 ± 0.0533	0.1201 ± 0.0378	0.1279 ± 0.0298	0.1280 ± 0.0388
Rapamycin	19	0.0981 ± 0.0279	0.1409 ± 0.0517$^{\&\&}$	0.1067 ± 0.0433*	0.1675 ± 0.0441	0.1421 ± 0.0100	0.2130 ± 0.0389$^{**\#}$	0.1114 ± 0.0290	0.1100 ± 0.0362	0.1866 ± 0.0480$^{**\#}$

Table 6: Effects of rapamycin on the expression of IL-2, FoxP3, and IL-10 in post-liver transplantation patients with ITBLs.

Data are presented as mean ± standard deviation.

$^{\$\$}$P<0.01 compared with pre-ITBL values in the control group.

$^{\&\&}$P<0.01 compared with pre-ITBL values in the rapamycin group.

*P<0.05 compared with ITBL values in the rapamycin group.

Interleukin-2 is mainly produced by lymphocytes, monocytes, and macrophages, and plays an important role in activating immune cells, mediating lymphocyte activation, proliferation, and differentiation,

and promoting the inflammatory response [12]. It is an important cytokine involved in the immune response and participates in anti-tumor effects and transplant rejection [13,14]. It has been found that there are a large number of regulatory T cells (Treg) with high expression of CD25 (CD4+, CD25 high) in transplant recipients. One of the important functions of Treg cells is to downregulate antigen-specific T cell responses, thereby improving graft-specific immune tolerance [15-17]. FoxP3 is a gene encoding transcriptional repressor protein, and FoxP3 mRNA and encoded protein are expressed specifically by Treg cells [18,19]. Therefore, it is a specific marker of Treg cells, and plays a crucial role in Treg cell function. IL-10 is mainly produced by TH2 cells, and is a cytokine with high biological activity that is involved in the regulation of multiple systems. In contrast to the biological effects of other interleukins except IL-8, IL-10 can inhibit the immune response via inhibiting the ability of TH1 cells and B cells to synthesize cytokines such as IFN-γ, IL-2, IL-3, TNF-β, and GM-CSF [20]. After activation, Treg cells can secrete a large amount of IL-10, thereby playing an important role in preventing autoimmunity, mediating transplantation tolerance, and regulating tumor immunity [21,22].

Tacrolimus is the first-line immunosuppressant after organ transplantation, reducing rejection and improving graft and recipient survival. However, it is also characterized by a narrow therapeutic window, and large individual differences in metabolism [23-27]. Indeed, after administration of "standard" doses, some patients show no therapeutic effects or serious side effects. Genetic factors such as polymorphisms can be closely related to drug metabolism. There is growing interest in the field of pharmacogenomics which focuses on the relationship between host genetics and drug metabolism [28]. Pharmacogenomics research on tacrolimus indicated that CYP3A enzymes, which are mainly expressed in liver and intestine, are the major metabolic enzymes of tacrolimus [29,30]. The rs776746 polymorphisms in intron 3 of CYP3A5 have been correlated with altered gene expression due to a splicing defect. These CYP3A5 rs776746 GG genotype carriers are associated with slow tacrolimus metabolism and higher concentration/dose (C/D ratio), while AG and AA genotype carriers associated with fast tacrolimus metabolism and lower C/D ratio [31-37].

The present study included subjects with complete follow-up records, and a series of liver biopsies. In addition to common non-specific inflammation, the liver biopsies also confirmed that ITBLs involve inflammatory cell infiltration in the portal area mixed with a large number of lymphocytes and monocytes, suggesting immunopathological damage. Histological manifestations included varying degrees of vacuolar degeneration of the bile duct epithelium, hyperplasia in a large number of small bile ducts, portal area expansion, and fibrosis. These changes gradually lead to dysfunction of the graft manifested as a significant increase in serum levels of ALT, AST, TBil, γ-GT, and ALP and a reduction in the serum levels of ALB, PALB, and CHE, eventually leading to hepatic decompensation and the need for retransplantation. We treated patients with ITBLs with rapamycin, and found that rapamycin can inhibit lymphocyte and mononuclear cell infiltration in the hepatic portal area, improve epithelial cell degeneration and hyperplasia in the bile duct, suppress immunopathological damage of the bile duct, and indirectly contribute to the restoration and healing of the bile duct epithelium, thus contributing to the recovery of liver function as indicated by reduced serum levels of ALT, AST, TBil, gamma-GT, and ALP and increased serum levels of ALB, PALB, and CHE. As a result, some patients with mild ITBLs remained in a stable condition or even achieved a positive clinical response. The possibility of liver retransplantation was therefore reduced.

The results of the present study showed that at the time of ITBL diagnosis, the average OD values of IL-2, but not FoxP3 and IL-10, in rapamycin-treated patients and controls were significantly increased compared with pre-ITBL values. This indicates that a large amount of the immune-activating cytokine IL-2 was released but the number of immunosuppressive FoxP3+ Treg cells and the amount of immunosuppressive cytokine, IL-10, did not increase accordingly. These changes can result in insufficient immune suppression, consequently leading to immunopathological damage of the bile duct and promoting the occurrence and development of ITBLs. After 6 months of treatment, there was no change in IL-2, FoxP3, and IL-10 expressions in the controls. However, rapamycin treatment led to a significant reduction in IL-2 expression and increase in FoxP3 and IL-10 expressions, suggesting that rapamycin can inhibit the expression of immune-activated IL-2, and promote the activity of FoxP3+ Treg cells and the expression of the immunosuppressive cytokine IL-10, thereby alleviating the immunopathological damage of bile ducts in ITBL patients. In the present study, all of 32 cases are CYP3A5, rs776746 allele A carriers, in which 15 cases have one alleles A, 12 cases two alleles A and 5 cases three alleles A totally in both donors and recipients. The study showed that, with increasing numbers of the alleles A, patients were found to have increasingly higher bile duct injury score at time points when ITBLs were identified. However, in all 40 cases who are CYP3A5, rs776746 allele A noncarriers in our center, there is no one suffering from ITBL. So it can be speculated that fast tacrolimus metabolism and lower concentration/dose (C/D ratio) might lead to sub-clinic rejection and immunopathological damage in bile ducts to some extent, which may be one of the pathogenesis of ITBLs after liver transplantation. In the follow-up study, we will include more cases to verify this speculation.

In conclusion, we believe that immunopathological damage, which maybe has an association with high expression of CYP3A5 rs776746 allele A, may be one of important pathological mechanisms of ITBLs. Rapamycin can alleviate the immunopathological damage in bile ducts to some extent, and indirectly contribute to the restoration and healing of the bile duct epithelium, thus contributing to the improvement of liver graft function and prevention of retransplantation in some patients with mild ITBLs.

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Author Contributions

Chuan-Yun Li was the first authors; he drafted the manuscript and statistical analyses. The experiments were designed by Shi-Chun Lu. The experiments were performed by Dong-Dong Lin, Dao-Bing Zeng, Qing-Liang Guo, Ju-Shan Wu, Ning Li directed the subject. Shi-Chun Lu supervised and coordinated the study. All authors have read and approved the final manuscript.

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