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Original Article

The role of myosin phosphatase target subunit 1 and related molecules in the destruction of common bile duct epithelium in children with pancreaticobiliary maljunction

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Abstract: Aim: To investigate the expression and potential significance of myosin phosphatase target subunit 1 (MYPT1), protein kinase C-potentiated inhibitor protein of 17 kDa (CPI17), and protein phosphatase 1 catalytic subunit (PP1c) in bile duct epithelium of children with pancreaticobiliary maljunction (PBM). Methods: Bile duct samples were collected from 22 children with PBM, and bile duct samples from 21 healthy cases served as controls. After immunohistochemical staining, the expression of MYPT1, CPI17, and PP1c and their corresponding phosphorylation in two kinds of tissues were assessed using image analysis system; meanwhile, their expression levels were measured using Western blot analysis. Results: Immunohistochemistry showed increased expression of P-MLC (P=0.001), P-CPI17 (P=0.013) and decreased expression of CPI17 and PP1c (P=0.000, P=0.012), MYPT1 (P=0.406) in the PBM group; Western-blot showed that in PBM group, the expression of p-MYPT1, p-CPI17, and MLCK were significantly increased (P<0.01), while the expression of MYPT1, CPI17, PP1c, and MLC were decreased (P<0.01). Conclusions: MYPT1, CPI17, and PP1c were expressed in bile duct epithelium, and the changes in their expression might be related to injury to the bile duct epithelial barrier in children with PBM.

Keywords: Pancreaticobiliary maljunction, epithelial barrier, MYPT1, CPI17, PP1c

Introduction

Pancreaticobiliary maljunction in children is a rare congenital anomaly, in which the main pancreatic and bile ducts are joined outside the duodenal wall, forming a markedly long and common channel. This channel results in the failure of the sphincter of Oddi in controlling the flow of bile and pancreatic juice and produces a continuous reflux of bile and pancreatic juice to each other [1]. Due to the reflux of bile and pancreatic juice, the bile contains a variety of pancreatin activators; trypsin and pancreatins (such as phospholipase A2) in bile duct are activated due to the long-term mixture of bile and pancreatic juice, which has strong destructive, corrosive, and digestive effects, and causes chronic inflammation of the bile duct wall and bile duct stones. This damages the tight junctions of the bile duct epithelium, decreases its barrier function, and further increases the probability of malignant transformation of bile duct epithelium [2].

One important step in the development of biliary tract diseases is the destruction of the biliary epithelial barrier, and the integrity of that barrier depends on the tight junctions (TJs) between epithelial cells [3-8]. In mucosal inflammation, disruption of the structure of the TJ mediates epithelial barrier dysfunction in two ways. First, inflammatory stimulation can induce the deficiency of specific types of TJ protein in the intercellular junction region [9, 10];

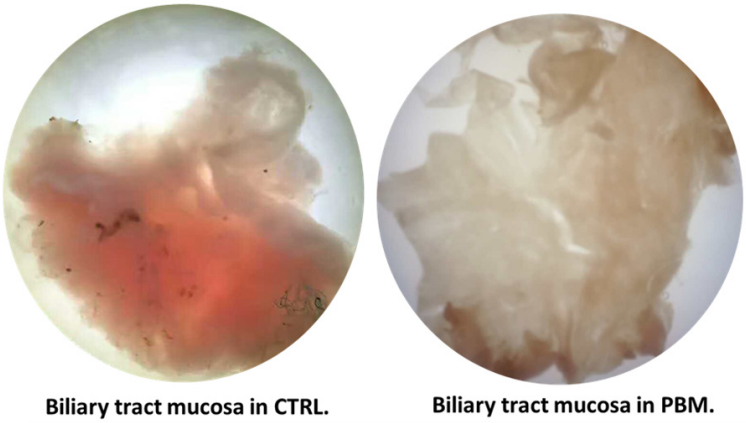

Figure 1. Biliary tract mucosa in PBM and CTRL.

second, the recombination of actin filaments plays an important role in the destruction of the epithelial TJs under inflammatory conditions [11].

Myosin II is a cytoskeletal motor that can convert chemical energy of ATP hydrolysis into mechanical energy to regulate the fixed tension and contraction of actin filaments [12]. Nonmuscle myosin II (NMII), expressed in epithelial cells, is a hexamer containing two heavy chains, two essential light chains (ELC), and two regulatory light chains (RLC). The activation of NMII requires phosphorylation of the Ser19 or Thr18/Ser19 residues of RLC, which leads to the assembly of actin filaments and self-assembly of NMII [12].

The phosphorylation of myosin light chain (MLC2) is determined by the relative activity of myosin light chain kinase (MLCK) and myosin light chain phosphatase (MLCP). The former phosphorylates MLC2, and the latter de-phosphorylates MLC2. Studies have shown that MLCK helps to regulate the dynamic structure and function of tight junctions; it could be activated due to the destruction of epithelial barrier [13, 14], can participate in the destruction of epithelial barrier of bile duct, and can promote the contraction of actin filaments. Unlike MLCK, MLCP can separate the MLC and actin by removing the phosphate group of p-MLC2 (Ser19) [15], and it can inhibit the contraction of actin filaments; therefore, it can be speculated that MLCP may also participate in the destruction of biliary epithelial barrier.

Research has shown that the activity of MLCP varies and is well regulated [16]. There are two main mechanisms underlying the regulation of MLCP: MYPT1, CPI-17, and their phosphorylated forms [17]. MYPT1 was a target subunit of MLCP, and its phosphorylated form, p-MYPT1 (Thr696), can inhibit the activity of MLCP. Multiple MYPT1 subtypes have been reported in several species and tissues [18, 19], and these MYPT1 subtypes are products of alternative splicing of a single gene [20, 21]. MYPT1 is expressed in both smooth muscle cells and non-muscle cells (including HeLa cells, HEK293 cells, and endothelial cells) [20-22]. PP1C is a catalytic subunit of MLCP, and it can enhance its own activity after binding to MYPT1 to regulate MLC2 phosphorylation [23]. One recent study reported that PP1C plays a regulatory role in endothelial barrier function [24]. CPI-17 was a MLCP specific phosphorylation-dependent inhibitory protein with molecular weight of about 17 kDa. The human CPI-17 encoding gene was located on chromosome 19q13.1, containing three introns and four exons, and was mainly expressed in trachea, uterus, and blood vessels.

Few studies had been done on the role of MLCP in the epithelial barrier. In this study, the changes in expression of MYPT1, CPI17, PP1c, and their corresponding phosphorylation in bile duct epithelia of children with PBM were studied. These efforts established the roles of MLCP in bile duct epithelia of children with PBM and provide experimental evidence for the further exploration of the molecular mechanism and regulatory targets of biliary epithelial barrier destruction in children with PBM.

Materials and methods

Sample characteristics

Bile duct samples from a total of 22 children with pancreaticobiliary maljunction and bile duct dilatation were selected from the Children's Hospital of Soochow University from 2015 to 2017. The magnetic resonance cholangiopancreatography (MRCP) or intraoperative cholangiography (IOC) of those children showed there to be common pancreaticobiliary channel joined outside the sphincter of Oddi, with over

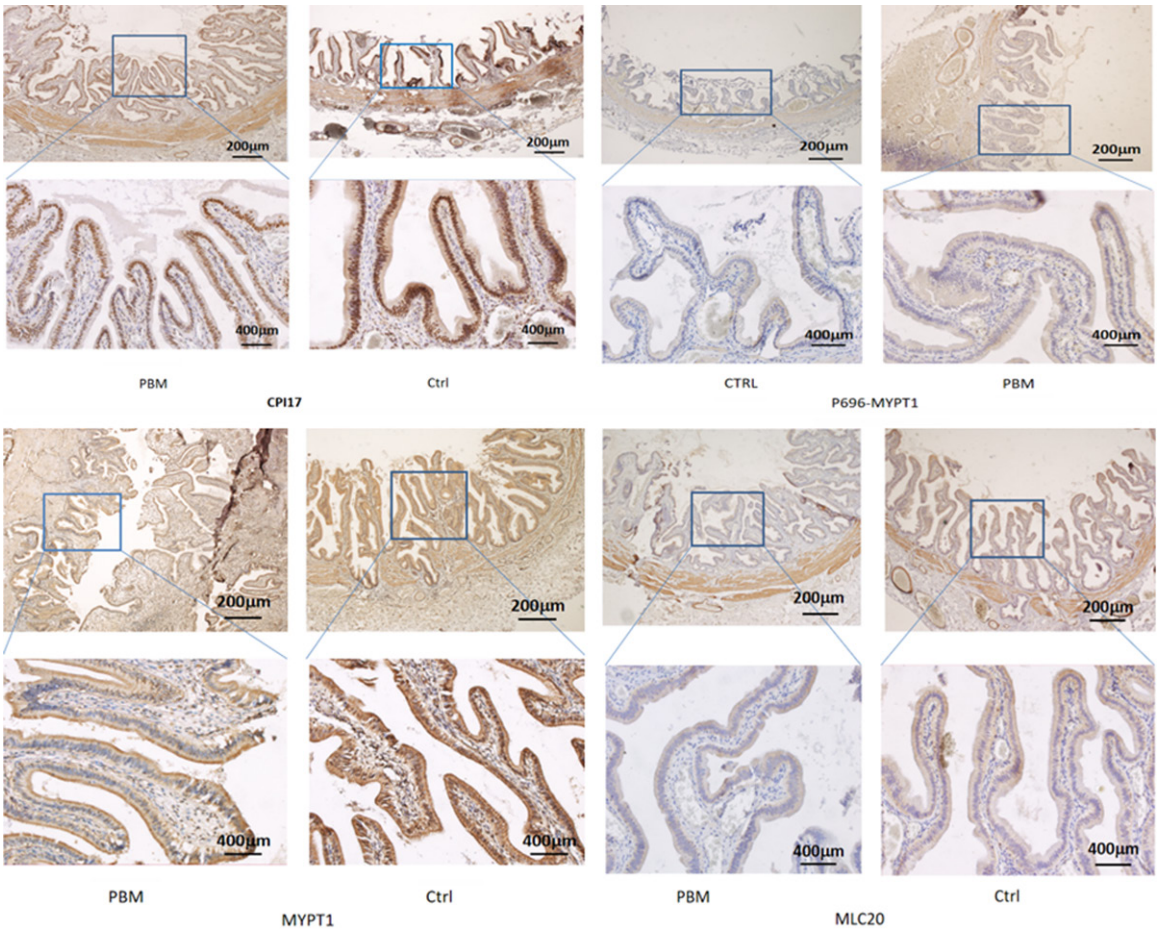

Figure 2. The comparison of immunohistochemical staining results of CPI17, MLC20, P696-MYPT1 and MYPT1 in human bile duct mucosa between PBM group and Ctrl group.

5 mm of length, and the biliary amylase activity over 1000 U/L. Bile duct tissues from 21 patients with cholelithiasis were used as controls, and no other biliary tract or pancreatic diseases were found in these patients. Samples were taken with the consent of the patients and their families, and the experiment was approved by the Ethics Committee of the Children's Hospital of Soochow University.

Experimental methods

Immunohistochemical method

The tissue samples were embedded in paraffin. Slices were cut to a thickness of 4 μm, incubated in 60°C incubator for 20 min before dewaxing, and then soaked in xylene twice and each for 10 min. Slices were then soaked in pure ethanol twice, each for 10 min; followed by 95% ethanol, 70% ethanol and 50% ethanol, 5 min each; at last, slices were soaked in distilled water for 5 min. Slices were placed in a 0.01 citrate buffer (pH 6.0), and placed in a microwave oven to keep the temperature of the liquid between 92 and 98°C for 10-15 min. The mixture was then cooled down for 30 min at room temperature. 3% H_2O_2 was added on the slice and blocked for 5-10 min to de-activate endogenous peroxidase. Slices were washed three times in PBS for 2-5 min each. Incubating the slices with serum from the same source as the secondary antibody in a humidor at 37°C for 15-30 min. We then discarded the blocking serum without washing; adding appropriate amount of primary antibodies, and put the slices in a humidor and incubated overnight at 4°C or 1-2 hours at 37°C. Slices were incubated at 37°C for 45 min, and washed by PBS for 3 times, 5 min each. At last, the color reaction was conducted using a ready-to-use immunohistochemistry kit (MaxVision^{TM}2 Kit 5920, anti-mouse/rabbit); DAB was used as chromogenic

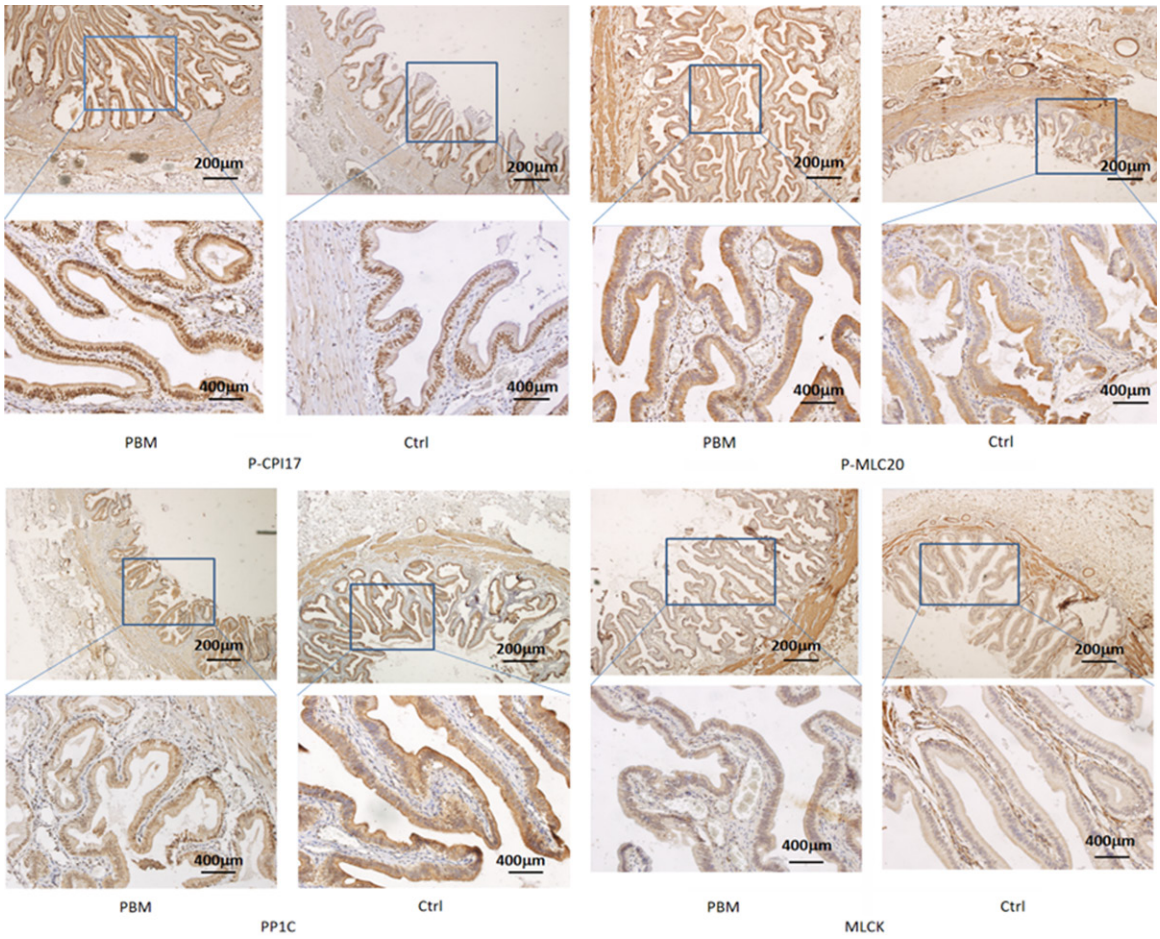

Figure 3. Immunohistochemical staining of P-CPI17, P-MLC20, PP1C and MLCK in human bile duct mucosa of PBM group and Ctrl group.

Table 1. Comparison of immunohistochemical staining scores of P-MLC protein

	Ctrl	PBM	P
Negative	0	0	0.001
Low positive	6	0	
Positive	14	15	
High positive	0	5	

Fisher's exact test. Notes: In the process of staining, 1 case in Ctrl group was unqualified, and 2 cases in PBM group were unqualified.

Table 2. Comparison of immunohistochemical staining scores of CPI17 protein

	Ctrl	PBM	X^2	*P*
Negative	0	0	15.785	0.000
Low positive	9	21		
Positive	11	0		
High positive	0	0		

Pearson chi square test. Notes: In the process of staining, 1 case in Ctrl group was unqualified, and 1 case in PBM group was unqualified.

reagent (quickly added, and observed the staining afterwards), and the color development was terminated according to the observations under microscopy (generally between 3-10 min, most commonly 5 min). Nucleus was stained using hematoxylin solution for 20-60 s; slices were then washed successively with water, 50%, 70% and 95% ethanol for 5 min each, followed by twice of 100% ethanol, 10 min each; finally, slices were soaked twice in xylene, 10 min each, and then sealed in neutral gum.

Scoring method

The overall score was given according to the percentage of positive cells (the average percentage of positive cells in five random field of

Table 3. Comparison of immunohistochemical staining scores of PP1C protein

	Ctrl	PBM	X^2	*P*
Negative	0	0	6.241	0.012
Low positive	3	11		
Positive	18	11		
High positive	0	0		

Pearson chi square test.

visions under 400 magnification) and the staining intensity (integral of the staining degree of most cells). The percentage of positive cells: 0%, scored 0; 0-25%, scored 1; 26-50%, scored 2; 51-75%, scored 3; ≥76%, scored 4. The staining intensity was scored according to the color depth: 0 for no color, 1 for light yellow, 2 for yellowish brown, and 3 for brownish brown. The overall score was calculated as the sum of both scores: 0 meant negative, 1-3 were weakly positive (+), 4-5 were moderately positive (++), and 6-7 were strongly positive (+++).

Western-blot

Under stereomicroscopy, normal saline was repeatedly injected between bile duct epithelium and muscular layer using 1 ml syringe until the structural layers became clear; then, the intact bile duct epithelium was isolated using tweezers (as shown in **Figure 1**). The total protein of bile duct epithelium was extracted and loaded on SDS gel, followed by steps such as electrophoresis, transfer to membrane, and blocking of the membranes. Then the PVDF membranes were incubated with polyclonal primary antibodies of MLC, p-CPI17 (Thr38), CPI17, p-MYPT1 (Thr696), MYPT1, PP1c, MLCK, and β-actin at dilution ratios of 1:1000, 1:1000, 1:1000, 1:1000, 1:1000, 1:1000, 1:500, and 1:2000, respectively, in sealed bags, shaken gently at room temperature for 2 h, and then allowed to incubate overnight at 4°C. The next day, the membrane was allowed to return to room temperature after gentle shaking for 1 h and washed 3 times using TBST buffer for 10 min each. Appropriate secondary antibody (1:5000) was used according to the source of the primary antibody and incubated with membrane for 1 h at room temperature with gentle shaking; after secondary antibody incubation, the membrane was washed three times using TBST buffer, 5-10 min each. Proteins were visualized using BeyoECL Plus Kit (Beyotime Biotechnology) and protein imaging system. The gray values of protein bands of MLC, p-CPI17 (Thr38), CPI17, p-MYPT1 (Thr696), MYPT1, PP1c, MLCK, and β-actin in each group were analyzed using ImageJ, and the gray values of each protein band were compared to the gray value of the internal standard, β-actin; the resulting ratios represented the relative expression levels of MLC, p-CPI17 (Thr38), CPI17, p-MYPT1 (Thr696), MYPT1, PP1c, and MLCK of each group.

Statistical analysis

The data were collected and sorted using Microsoft Excel and statistically analyzed using SPSS. The measurement data are here expressed as mean ± standard deviation and compared using the independent sample t test or Wiloxon rank-sum test. The counting data and percentages were compared using the chi-square test and Fisher's exact test. $P<0.05$ was considered statistically significant.

Results

Immunohistochemical staining

The results showed that MLCK, MYPT1, CPI17, and PP1c were mainly expressed in the epithelial cytoplasm; the expression of MYPT1, CPI17, and PP1c was lower in the bile ducts of children with PBM than in those of controls, and the expression of MLCK, P-MYPT1 (Thr696) and P-CPI17 (Thr38) was higher (**Figures 2** and **3**). Immunohistochemistry showed increased expression of P-MLC ($P=0.001$), P-CPI17 ($P=0.013$) and decreased expression of CPI17 and PP1c ($P=0.000$, $P=0.012$), MYPT1 ($P=0.406$) in the PBM group (**Tables 1**-**3**, **5** and **6**).

Western-blot

Western-blot analysis showed the expression of MYPT1, CPI17, and PP1c in PBM to be significantly lower in the bile ducts of children with PBM than in those of controls ($P<0.01$), and the expression of MLCK, p-MYPT1 (Thr696), and p-CPI17 (Thr38) was significantly higher ($P<0.01$); the changes in expression were consistent with the results of immunohistochemical staining. Western-blot results are shown in **Figure 4**, and the relative expression levels are shown in **Table 4**.

Table 4. The expression ratio of MLC, p-CPI17 (Thr38), CPI17, p-MYPT1 (Thr696), MYPT1, PP1C and MLCK by western blot

	Relative protein expression ($\bar{x} \pm s$)		Z	P
	PBM (n=15)	Ctrl (n=10)		
MLC2	0.1807±0.01263	3.2677±0.2779	-4.160	<0.01**
p-CPI-17 (Thr38)	0.4546±0.03757	0.1438±0.02542	-3.938	<0.01**
CPI17	0.2457±0.01619	1.4623±0.2142	-4.160	<0.01**
p-MYPT1 (Thr696)	1.1873±0.14044	0.2376±0.04844	-4.160	<0.01**
MYPT1	0.459±0.054	1.138±0.189	-3.162	<0.01**
PP1c	0.202±0.032	0.853±0.120	-4.049	<0.01**
MLCK	0.790±0.058	0.371±0.039	-3.716	<0.01**

**Wilcoxon two-sample test.

Table 5. The comparison of immunohistochemical staining scores of P-CPI17 Protein

	Ctrl	PBM	P
Negative	1	1	0.406
Low positive	5	9	
Positive	14	10	
High positive	0	0	

Fisher's exact test. Notes: In the process of staining, 1 case in Ctrl group was unqualified.

Table 6. The comparison of immunohistochemical staining scores of MYPT1 protein

	Ctrl	PBM	P
Negative	5	0	0.013
Low positive	14	16	
Positive	1	6	
High positive	0	0	

Fisher's exact test. Notes: In the process of staining, 1 case in Ctrl group was unqualified, and 2 cases in PBM group was unqualified.

Discussion

In this study, we found that, in the bile duct epithelia of children with PBM, the expression of MLCK and p-MLC2 (Ser19) were higher than those in the control group, while the expression of MLC2 was lower; these results were consistent with a recent study by Guo [25, 26]. It had been shown that the contraction of myosin filaments at junctions induced by MLCK to be an important mechanism of epithelial barrier destruction in inflammatory condition. This mechanism was important for the destruction of LPS-induced paracellular barrier in intestinal, pulmonary epithelial and bile duct monolayers [9]. The studies by Andreeva and Banan further confirmed that the destruction of the tight junctions of bile duct epithelium was closely related to the inflammatory condition mediated by LPS and hydrogen peroxide, and this destruction could be inhibited by MLCK inhibitors [27, 28]. For this reason, we here speculate that the chronic inflammation of the biliary epithelia of children with PBM might cause the destruction of epithelial barrier by increasing MLCK expression, which up-regulates MLC2 phosphorylation.

We also found that the expression of MYPT1 and PP1c in bile duct epithelium of children with PBM were lower than those in control group, while the expression of p-MYPT1 (Thr696) was higher. In smooth muscle cells and fibroblasts, MYPT1 was mainly distributed in the cytoplasm [20, 29]. Other studies reported that MYPT1 was expressed in the cell-cell junctions between endothelial cells [30], and the distribution of MYPT1 was co-located with F-actin. Similarly, MLCK and p-MLC2 (Ser19) were also found to be co-located in cell-cell junctions [31, 32]. These results suggested that MYPT1 might be involved in the regulation of barrier function by regulating MLC2 phosphorylation. Recent studies also supported our hypothesis that MYPT1 is involved in the regulation of endothelial cell barrier function [20, 33]. He et al. [34] also reported that MYPT1 is expressed in intestinal epithelial cells and might be involved in the regulation of intestinal epithelial barrier function in inflammatory bowel diseases. MYPT1 was the target subunit of MLCP, which could target and bind p-MLC2 (Ser19) and reduce the phosphorylation of MLC2; meanwhile, MYPT1 contained several phosphorylation sites [35], which could be spe-

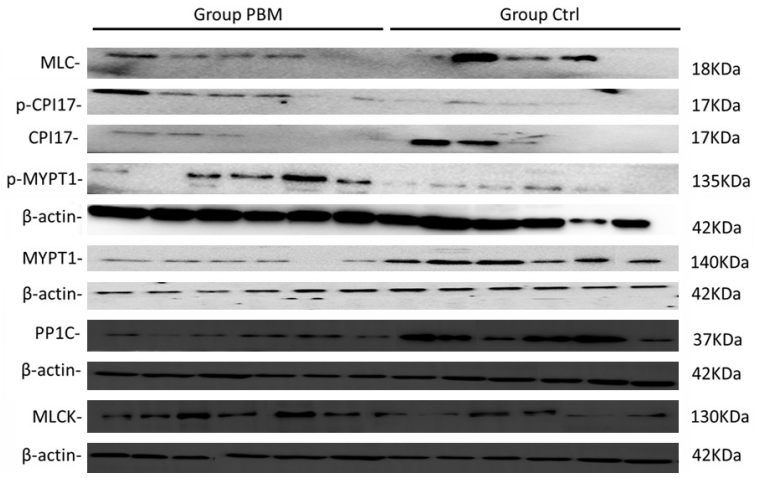

Figure 4. Western-blot strip diagram of MLC, CPI17, p-CPI17, MYPT1, P-MYPT1, PP1C and MLCK in human bile duct mucosa of PBM group and Ctrl group. (The first two lines of P-MYPT1 decreasing in Group PBM as compared to Group Ctrl because of Sample error).

cifically phosphorylated by ROCK, forming p-MYPT1 (Thr696) and so inhibiting MLCP activity. This study showed that, in the biliary epithelium of children with PBM, the expression of MYPT1 was decreased, while the expression of p-MYPT1 (Thr696) was increased. The lack of targeted binding opportunities for p-MLC2 led to the failure of p-MLC2 de-phosphorylation, which resulted in the enhanced contraction of actin and myosin filaments around the epithelial junctions of the biliary tract in children with PBM, followed by tensions on the tight junctions and the cell surfaces. The tight junctions were then opened, and the epithelial barrier of the biliary tract was destroyed. There was an ankyrin repeat in the N-terminal of MYPT1, consisting of 7 or 8 anchor repeats and could be used as binding sites for multiple ligands [36]. The N-terminal of the first anchor repeat in the flank side was PP1c binding motif, the RVXF motif [37]. PP1c is the catalytic subunit of MLCP with a molecular weight of about 38 kDa. It can enhance its own activity after binding with MYPT1 and de-phosphorylate pMLC20 through the targeted binding of MYPT1 to pMLC20. In this study, the expression of PP1c in the bile duct epithelium of children with PBM was decreased, and the decreased PP1c expression might further lead to the failure of p-MLC20 de-phosphorylation, which can strengthen the contraction of the contractile actomyosin ring, open the tight junctions, and aggravate the destruction of epithelial barrier.

In this study, we assessed the expression of CPI-17 and p-CPI-17 in the bile duct epithelium of children with PBM and the control group and confirmed the significantly lower levels of CPI-17 expression and significantly higher levels of p-CPI-17 expression in the bile duct epithelia of PBM children, assessed at the protein level. CPI-17 had an inhibitory effect on MLCP activity, and the activity of CPI-17 was mediated by the Rho/ROCK pathway, which could also re-phosphorylate CPI-17 by activating PKC [38]. CPI-17 has a unique V-shaped structure without phosphorylation, consisting of paired A/D and B/C four-helix bundle, and there was found to be a threonine in position 38 of the gap between helical bundles. After phosphorylation of the threonine in the position 38 of CPI-17, the phosphorylated residue was exposed to the protein surface, limiting the hydrolysis of MLCP. The inhibition of the phosphorylated residue was more than 1000 times more pronounced than that of the non-phosphorylated residue [39]. Other studies found that phosphorylated CPI-17 could bind closely to the N-terminal α helix of MYPT1 and promote their interaction with each other [39], which might be why phosphorylated CPI-17 could specifically inhibit the activity of MLCP. Because the MLCP inhibition activity of p-CPI17 was significantly enhanced, which led to the decrease of MLC20 de-phosphorylation and the increase of overall expression of p-MLC20, the tight junctions were opened and the epithelial barrier was damaged; so the increased phosphorylation of CPI-17 might also be involved in the destruction of biliary epithelial barrier in children with PBM.

In conclusion, by studying the changes in expression of MYPT1, PP1c, and CPI-17 and their phosphorylation in bile duct epithelia of children with PBM, the following conclusions were drawn: the lower expression of MYPT1 and higher expression of p-MYPT1 (Thr696) in PBM group led to the decreased de-phosphorylation ability of MLC2. The total level of MLC2 phosphorylation [p-MLC2 (Ser19)] was greater under the synergistic action of p-CPI17 and

PP1c, which resulted in the increased contraction force of the contractile actomyosin ring around the junctions of bile duct epithelium in children with PBM, followed by tensions on the tight junctions and cell surfaces, and eventually the destruction of the epithelial barrier.

Disclosure of conflict of interest

None.

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