

Original Article

Expression of CTGF and BMP7 in experimental murine biliary atresia

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Abstract: Biliary atresia (BA) is a rapidly progressive disease in neonates characterized by fibro-obliteration of the extrahepatic biliary tree that results in chronic cholestasis and biliary cirrhosis. Although many studies have been conducted to explore the progressive mechanisms in and reasons for this disease, its etiology still remains unclear. Previous studies have suggested that epithelium-mesenchymal-transition (EMT) was involved in the progression of fibrosis and eventual disappearance of the biliary tract. It has been confirmed that the TGF- β 1-SMAD-BMP7 signaling pathway is an important inducing factor in the EMT process, while CTGF and BMP7, which are important effective factors of this signaling pathway, contribute to EMT progression and fibrosis, respectively. In this study, we studied the expression of CTGF and BMP7 in the extrahepatic bile duct and liver tissue of rhesus rotavirus (RRV) by inducing biliary atresia in mouse models and explored the potential roles of CTGF and BMP7 in the development of BA. Methods: BALB/c mice were infected with RRV intraperitoneally within 24 hours after birth, while the control group was administered saline. The animals were observed, and their general condition was recorded. The animals were sacrificed on day 1, 3, 5, 7, 10, 15, and 20 after inoculation; extrahepatic bile ducts and liver tissues were collected; the CTGF and BMP7 expression levels in the BA and control groups were detected with immunohistochemistry; and real-time quantitative RT-PCR and Western blot results were compared between the BA group and normal control using Student's *t* test. Results: The animals that were inoculated with RRV displayed symptoms of cholestasis beginning on the fifth day of life, including jaundice, grey stool, oily fur, lethargy, and growth retardation. CTGF expression in the BA group increased shortly after injection and continued at high levels; the BMP7 expression increased after CTGF, and the difference between the two groups became less evident beginning on day 15. Conclusions: CTGF and BMP7 expression in the hepatobiliary tissue of MMU18006-exposed mice significantly increased between the BA and control groups, indicating their potential role in the pathogenesis of BA; the duration of high BMP7 expression was shorter than that of CTGF that suggested the anti-fibrosis role was limited.

Keywords: Biliary atresia, rhesus rotavirus, CTGF, BMP7

Introduction

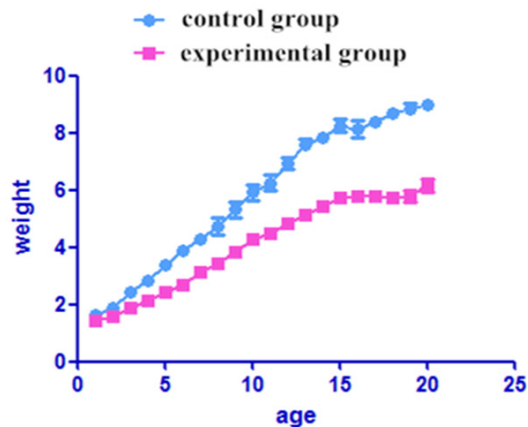
Biliary atresia (BA) is known to be a devastating liver disease of unknown etiology affecting infants generally within the first 3 months of life. The main manifestation of this disease is characterized by inflammation, obstruction, and subsequent obliteration of the extrahepatic bile ducts, fibrosis, and liver failure. Without surgical intervention, most infants develop cirrhosis within months of disease onset. The mechanisms responsible for disease pathogenesis are not fully understood [1-6]. Bile duct inflammation or injury may be potential inciting factors that lead to progressive inflammation and fibrosis of the extra- and intrahepatic biliary tract; the lesion persisted even after suc-

cessful hepatoportoenterostomy (HPE) and bile drainage. A viral infection induces an autoimmune reaction against the biliary tract that is believed to be one of the reasons for biliary tract damage [7-14]. A number of factors involved in the TGF β -SMAD-BMP7 signaling pathway have been implicated in the development and progression of biliary atresia through processes such as EMT [15, 16].

Manifestations of biliary atresia have been induced by inoculating BALB/c mice with rhesus rotavirus (RRV); and this RRV-induced animal model has been used for the study of histological and biochemical features of extrahepatic biliary atresia, enabled mechanistic studies and uncovered molecular, cellular and gene-

Table 1. Details of antibodies

Reagents	Product Number	Company
Antibody of CTGF	Ab6992	Abcam
Antibody of BMP7	Ab56023	Abcam
Goat anti-rabbit IgG	A0277	Beyotime

**Figure 1.** Weight difference between the experimental and control groups.

expression patterns that define experimental biliary atresia, and identified key initiating and progression events that result in extrahepatic biliary obstruction [17-21].

CTGF and BMP7 are factors in the TGFbeta-SMAD-BMP7 signaling pathway. Expression of CTGF may induce production and deposition of collagen, while BMP7 may alleviate fibrosis. Roles of TGF beta in the progression of BA have been elaborated; in this study, we explored the temporal expression of CTGF and BMP7 in the extrahepatic bile ducts or liver tissues of animal models with BA, to examine the possible roles of CTGF and BMP7 in the progression of fibrosis.

Materials and methods

Cells and viruses

The RRV strain MMU18006 was purchased from ATCC and propagated on MA104 as previously described [20]. Briefly, the RRV titer of the supernatant was 1.0×10^6 plaque forming unit (pfu) per ml. To prepare purified virus, virus-infected cells were harvested after a 50-percent to 80-percent cytopathic effect was attained.

RRV-induced animal model of BA

Pregnant BALB/c mice were purchased from the Center Laboratory of Shengjing Hospital, China Medical University. Newborn BALB/c mice were injected intraperitoneally (i.p.) with 50 μ l of RRV of 1.0×10^6 pfu/ml or Hank's balanced saline solution as a control within 24 hours of life. The extrahepatic bile duct and liver were harvested on day 1, 3, 5, 7, 10, 15, and 20. Ethical approval was obtained from the China Medical University Animal Ethics Committee prior to the start of the study.

Histology and immunostaining

Formalin-fixed, paraffin-embedded bile ducts were sectioned at 3 μ m, stained with hematoxylin and eosin (H&E), or further processed for immunostaining. Immunohistochemical (IHC) staining was performed according to the protocols provided by the manufacturers. Slides were incubated at 4°C overnight with antibodies to CTGF or BMP7; the samples were then incubated with goat anti-rabbit IgG (Table 1) for 10 min at room temperature. DAB (Sigma Chemical Co., St. Louis, MO) was precipitated and developed under a light microscope, and the sections were counterstained. The specimens were mounted and imaged using a digitized microscope camera (Nikon E800, Japan).

Isolation of liver RNA and RT-PCR

Total RNA was isolated from liver tissues with Trizol reagent (Invitrogen) according to the manufacturer's protocol. RNA (1 μ g) was reverse-transcribed using a PrimeScript RT reagent kit (TaKaRa) according to the manufacturer's instructions. Quantitative real-time PCR was performed using an SYBR Green PCR Master Mix (SY1020, Solarbio, China) on a 7900HT fast real-time PCR system (Applied Biosystems) under the following conditions: 95°C for 10 min, 40 cycles of 95°C for 10 s, 60°C for 20 s, 72°C for 30 s, and 4°C for 5 min. A dissociation procedure was performed to generate a melting curve for confirmation of amplification specificity. The housekeeping gene β -actin (Takara, code D3783) was used as an endogenous control. The relative gene expression levels were determined using Δ Ct = Ct gene - Ct reference, and fold changes in gene expression were calculated using the $2^{-\Delta\Delta$ Ct method. Experiments were repeated in triplicate. Primer sequences

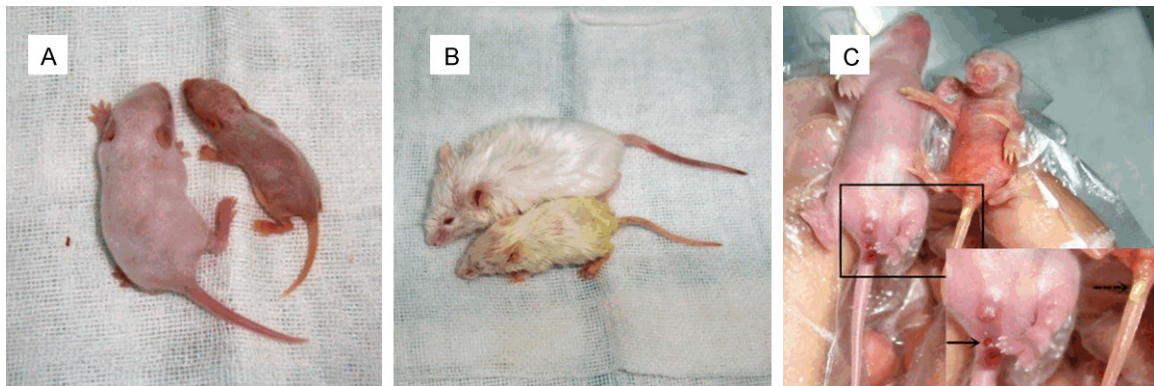


Figure 2. Mice with cholestasis and their stool. Mice on 10 d (A) and 20 d (B); jaundice and retardation could be observed, as compared with the control; oily fur (B) and clay-like stool (C) were observed.

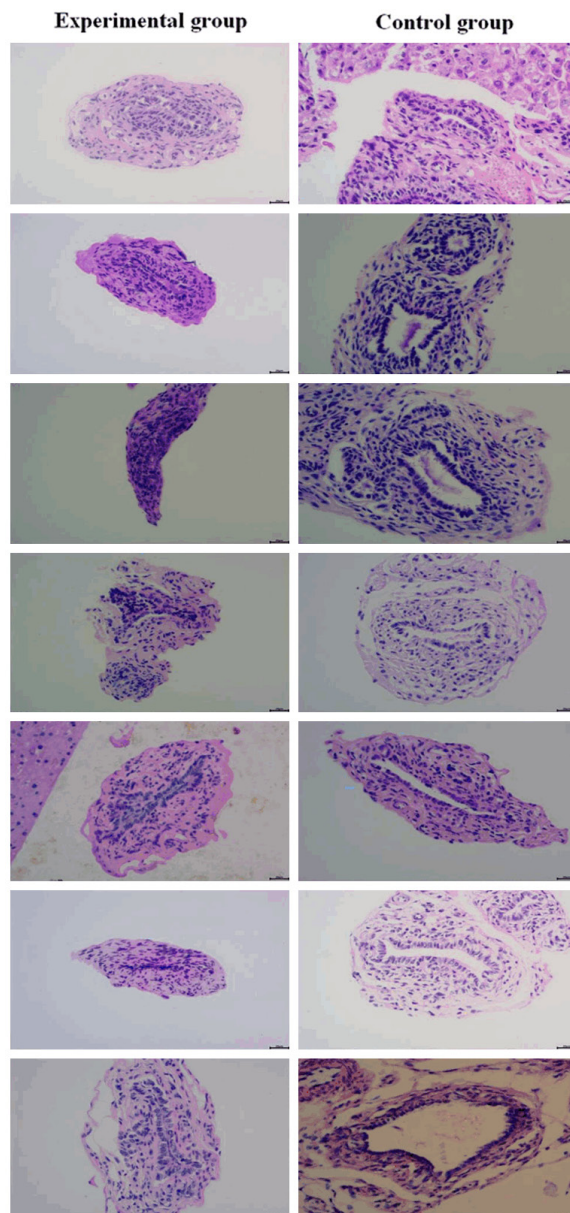


Figure 3. H&E staining of the common bile ducts in the experimental and control groups. Inflammatory infiltration, stenosis, and atresia of the common bile ducts could be seen beginning on day 3 (200 \times).

spanning the intron-exon junction were as follows: BMP7 forward, 5'-CTCCAAGACGCCAAAGAACC-3', reverse, 5'-CCTCACAGTAGTAGGCAGCATA-GC-3'; CTGF forward, 5'-AGCCAGGA-AGTAAGGGACACG-3', reverse, 5'-CGTTCTCACTTTGGTGGGATAG-3', β -actin forward, 5'-TTTTCAGCCTTCTTCTT-GGGTAT-3', reverse, 5'-CTGTGTTGGC-ATAGAGGTCTTTACG-3'.

Protein preparation and Western blot analysis

Protein was prepared from the liver tissues on day 1, 5, and 15 after injection according to previously described methods: the liver tissues were pooled and sonicated in ddH₂O-containing protease inhibitors. Forty-microliter protein extracts were heated at 90°C for 10 min and size-fractionated on Bis-Tris SDS-PAGE gels (Invitrogen, CA). Protein samples were denatured, separated using SDS/PAGE, and transferred onto PVDF membranes (Millipore, Billerica, MA); were blocked with 5% fat-free milk in Tris-buffered saline (1 h, RT); and were incubated overnight at 4°C with a primary antibody against CTGF or BMP7 (diluted 1:2000). The membrane was then incubated with secondary antibody

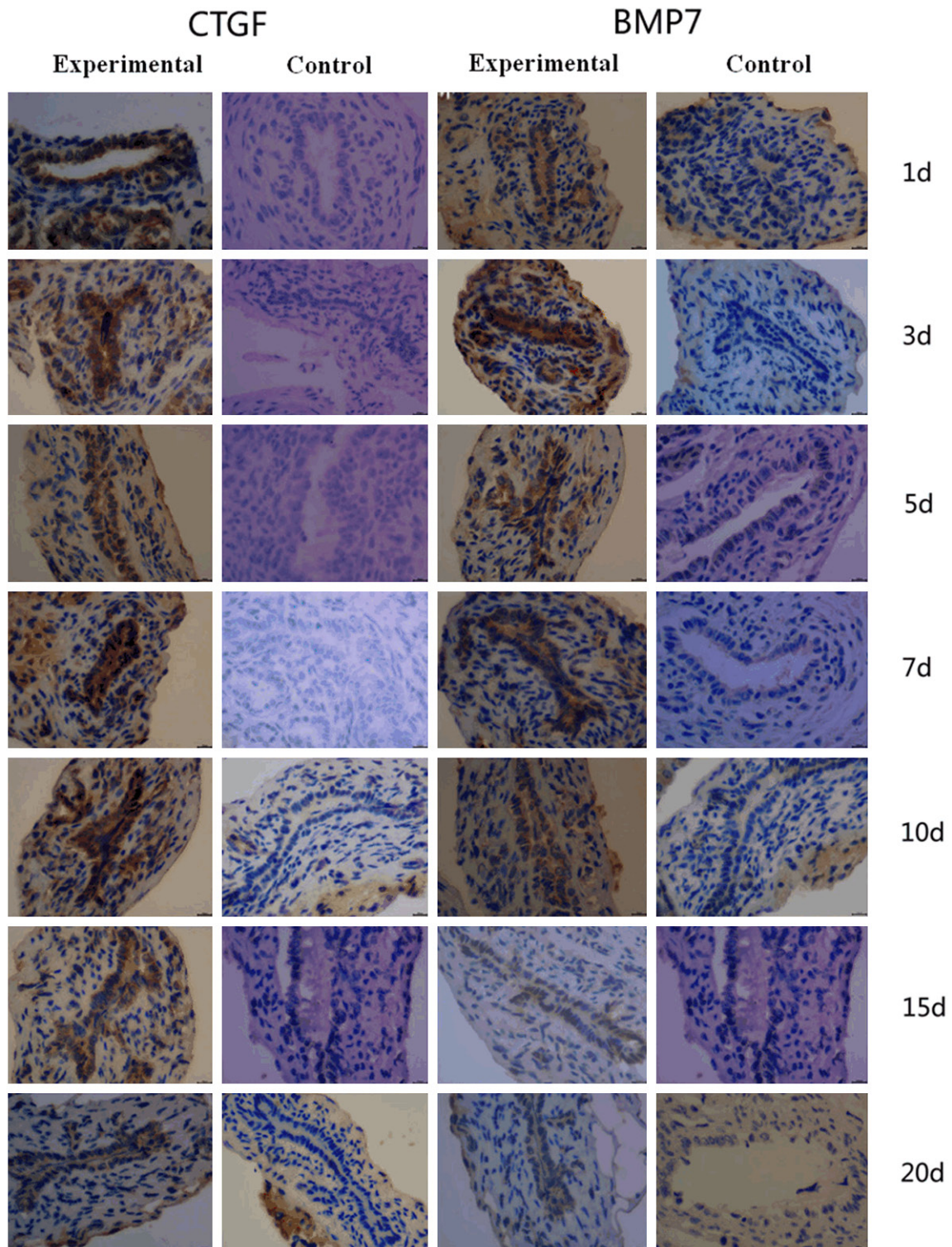


Figure 4. CTGF and BMP7 expression in the experimental and control groups. The immunoreactivity for both CTGF and BMP7 was higher than that of the control; differences in BMP7 were more evident during the early stage (1-10 days) than the later stage (15-20 days) (400 \times).

(diluted 1:5000), and immunostained bands were detected with a ProtoBlot II AP System

with a stabilized substrate (Promega). β -Actin protein was used as an internal control.

Table 2. Relative CTGF mRNA in liver samples from the experimental and control groups

Time point	N	Experimental group	Control group	P
1 d	10	1.14±0.04	1.00±0.02	<0.05
3 d	10	1.31±0.06	0.81±0.15	<0.05
5 d	10	1.79±0.03	1.34±0.06	<0.05
7 d	10	1.48±0.02	0.78±0.01	<0.05
10 d	10	2.95±0.06	0.76±0.03	<0.05
15 d	10	3.02±0.07	2.14±0.06	<0.05
20 d	10	3.34±0.11	1.27±0.02	<0.05

Table 3. Relative BMP7 mRNA in liver samples from the experimental and control groups

Time point	N	Experimental group	Control group	P
1 d	10	1.00±0.01	0.96±0.35	>0.05
3 d	10	0.75±0.45	1.36±0.03	-
5 d	10	2.02±0.08	1.36±0.03	<0.05
7 d	10	1.38±0.03	1.50±0.06	-
10 d	10	2.46±0.12	1.93±0.02	<0.05
15 d	10	5.25±0.13	3.28±0.09	<0.05
20 d	10	4.02±0.07	3.42±0.06	<0.05

Results

Manifestations of the animals

BALB/c mice inoculated with RRV within 24 hours of age and displayed symptoms of cholestasis including growth retardation, anepithymia, jaundice, clay-like stool, and oily fur. Their manifestations became more obvious with time. Mice in the control group exhibited none of these symptoms (**Figures 1, 2**).

Hematoxylin and eosin staining of the extrahepatic bile duct

Inflammatory filtration around the extrahepatic bile ducts, along with stenosis and atresia of the common bile ducts, could be detected beginning on day 3 (**Figure 3**).

Immunohistochemical staining

In the experimental group, the common bile duct epithelium exhibited immunoreactivity for both CTGF and BMP7 on the first day after inoculation; the immunoreactivity of both CTGF and BMP7 were increased, as compared with the

control group (**Figure 4**). Differences in BMP7 became less evident beginning on day 15.

Real-time RT-PCR

The OD value for total RNA was calculated by A260/A280 and was from 1.8 to 2.0. The CTGF and BMP7 expression levels were normalized to the mRNA level of β -actin from the same specimen. Consistent with the results from the immunohistochemical study, the CTGF and BMP7 expression in mRNA levels was evaluated using RT-PCR and are summarized in **Tables 2 and 3**; the expression of CTGF mRNA was significantly elevated in the experimental group, as compared with the normal control at each time point, while the expression of BMP7 slowly increased.

Western blot analysis

A Western blot analysis specific for CTGF and BMP7 was performed to quantify protein expression in the liver tissues on day 1, 5, and 15 for both the experimental and control groups. As for protein extracted from both the experimental and control groups, CTGF and BMP7 were detected as approximately 36-kDa and 49-kDa bands, respectively on Western blot. A corresponding β -actin band was normalized against each protein band. We noticed that the CTGF expression gradually increased from day 1, as compared with the normal control (**Figure 5**); the same tendency was also noted with BMP7, but the difference in BMP7 became less evident in the 15-day group (**Figure 6**).

Discussion

BA is a rare disorder in young infants with an unknown etiology; it is characterized as fibro-inflammatory obstruction and obliteration of extrahepatic bile ducts in the first few weeks of life [1-7]. BA has been recognized as one of the most common causes of neonatal cholestasis and one of the most common reasons for liver transplantation (LT) in childhood. If untreated, it rapidly progressed to biliary cirrhosis that could be fatal within the first year. Since the induction about 50 years ago, Kasai operation has been widely recognized as the first choice for the treatment of biliary atresia; a successful Kasai operation could restore biliary outflow, reduce cholestasis and cirrhosis, dramatically change the outcome of biliary atresia, and

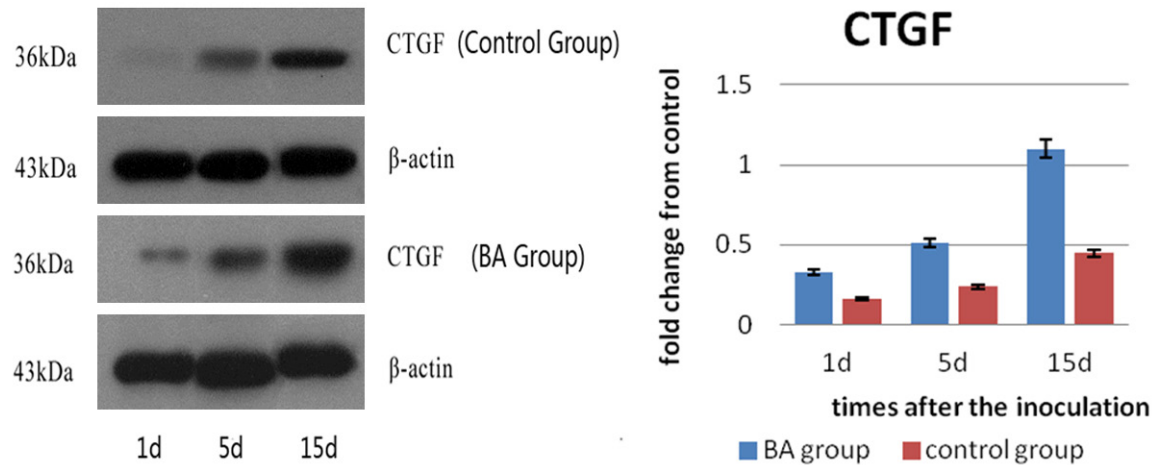


Figure 5. Western blot results of CTGF.

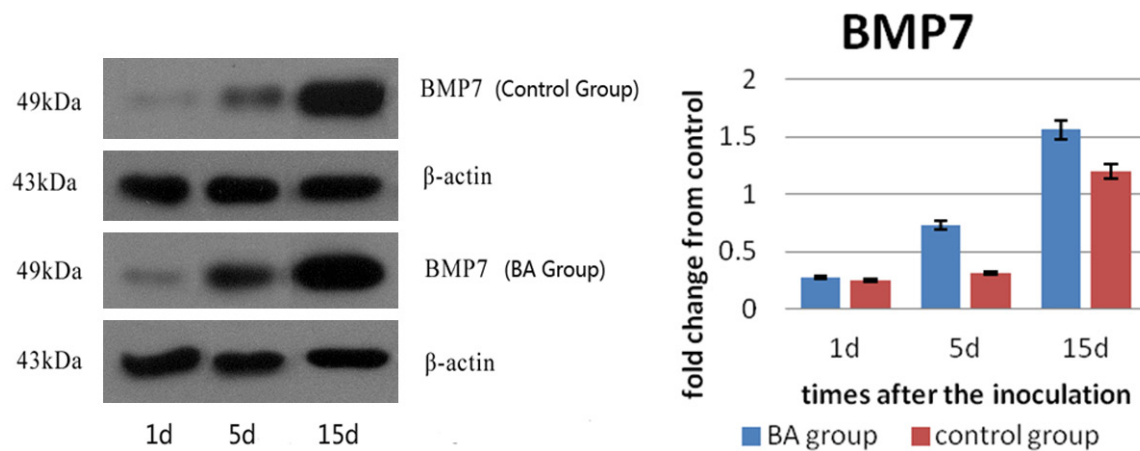


Figure 6. Western blot results of BMP7.

improve the overall survival rate of the native liver. However, in some cases, even after successful bile drainage and clearance of jaundice, ongoing injury to the intrahepatic bile ducts and progressive fibrosis could last and eventually lead to end-stage cirrhosis and the need for LT in approximately 50% of affected children [22-24].

It has been accepted that the outcomes of hepatoportoenterostomy are highly related to the timing of the operation; children who underwent an operation under 3 months of age may achieve satisfying outcomes. One study in France estimated that 5.7% of all LTs might be avoided if all BA patients underwent a Kasai operation before 46 d of age. Another study in Taiwan found that the LT rate was lower in BA patients who underwent a Kasai operation

within 60 d of age than those who underwent the operation after 60 d of age [25].

Tissue has the potential to self-repair after injury through fibrosis; liver fibrosis is a dynamic process characterized by the accumulation of extracellular matrix (ECM) resulting from the wound-healing response in liver tissues secondary to liver injury from any etiology. Ongoing liver fibrosis reflects an imbalance that favors fibrotic progression (fibrogenesis) over regression (fibrolysis). Although liver fibrosis may be relieved in some patients after a successful Kasai operation; but in the others fibrosis will last and lost the potential of reversibility. During the progression of fibrosis, many molecular factors are involved in the TGFβ-Smad-BMP7 signaling pathway that play important roles by regulating a broad range of cellular responses

including proliferation, differentiation, migration, and apoptosis; dysfunction in signaling regulation has been implicated in various human diseases including cancer, fibrosis, autoimmune and vascular disorders, and biliary atresia [26-32]. CTGF and BMP7 are both important factors in the of TGF β pathway. CTGF is an important factor in promoting myofibroblast differentiation and enhancing ECM synthesis, while BMP7 is an important anti-fibrotic cytokine that plays a crucial role in anti-fibrosis and suppressing EMT progression. Biliary atresia was characterized by progressive fibrosis of liver tissue even after a successful operation.

In this study, we investigated the spatial and temporal expression pattern of CTGF and BMP7 in a biliary atresia mouse model using immunohistochemistry staining, Western blot, and qRT-PCR analysis. We found that the expression of CTGF in bile duct cells and liver tissues increased shortly after injection; BMP7 expression increased after CTGF, and the increase in BMP7 did not last as long as that of CTGF, with a significant difference demonstrated between the experimental and control groups. These manifestations revealed that CTGF and BMP7 were involved in the development of biliary atresia, inflammation, and collagen deposition; when the fibrosis mechanism was activated after any injury, the anti-fibrosis mechanism was also activated to keep a balance between fibrosis and fibrolysis, but with the aggravation of fibrosis, the antagonistic mechanism will be exhausted eventually. As for the treatment of biliary atresia, it is well known that operative timing is important for clinical outcomes; there should be a time point within the first 3 months, but when should it be? Are there any biomarkers? Although many factors involved in biliary atresia have been identified in recent years, their regulatory mechanisms, as well as interactions between different factors, remain poorly understood. Further analysis of the cross-talk between different signaling pathways and cytokines might provide a greater appreciation of the developmental process and might facilitate a better understanding of the pathogenesis of biliary atresia.

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Disclosure of conflict of interest

None.

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