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ORIGINAL ARTICLE

RETINOIC ACID-INDUCIBLE GENE-I AND CXCL10 ARE INVOLVED IN BILIARY ATRESIA

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Abstract ***Purpose:*** Retinoic acid-inducible gene-I (RIG-I) is a member of cytoplasmic viral sensors which plays an important role in inflammation of biliary epithelial cells (BECs). The aim of this study is to examine if RIG-I and C-X-C motif chemokine 10 (CXCL10) are involved in the etiology of human biliary atresia (BA).

Methods: Immunohistochemical study was performed on surgically resected tissues obtained (June 1994 to March 2016) from 30 infants with BA and non-inflamed hepatic tissues from 7 infants with hepatoblastoma. A semiquantitative scoring system was designed to evaluate the staining with an antibodies to the RIG-I and CXCL10. The expression of RIG-I and CXCL10 in HuCCT1 cholangiocarcinoma cell line were studied by western blotting, ELISA and RT-PCR analyses.

Results: Intense immunoreactivity for RIG-I and CXCL10 was detected in BECs in tissues resected from BA patients. The expression of RIG-I and CXCL10 in the hilar tissue was significantly stronger than in the hepatic tissue. Transfection of HuCCT1 cells with poly(I:C), a synthetic analog of viral dsRNA, induced the expression of RIG-I, and knockdown of RIG-I inhibited the induction of CXCL10 in HuCCT1 cells transfected with poly(I:C).

Conclusion: These results suggest that RIG-I-CXCL10 cascade may be involved in the etiology of human BA.

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Key words: biliary atresia; retinoic acid-inducible gene-I; CXCL10; HuCCT1; poly(I:C).

Introduction

Biliary atresia (BA) is a rare disease in infants which is characterized by progressive inflammation and fibrous obstruction of both the extrahepatic and intrahepatic bile ducts. Similar pathological process does not exist in older children or adults. If untreated, it has fatal consequences to child health with rapid progression to end-stage cirrhosis. Good outcomes depend on early diagnosis and timely Kasai portoenterostomy.

The cause of BA is not known, but recent studies suggest an autoimmune process. Currently it is thought that both the primary perinatal hepatobiliary viral infection and the secondary generation of an autoimmune-mediated bile duct injury are involved in the etiology of BA[1,2)]. When the cells are infected with viruses, pathogen-associated molecular patterns (PAMPs) of viruses are recognized by pattern recognition receptors, and innate immune reactions against viruses are initiated. Innate immune reactions and subsequent inflammation

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are important for host defense, but dysregulated inflammation may lead to tissue injury. Since initially suggested by Landing in 1974[3], several viruses have been implicated in the etiology of BA. Reoviridae, a kind of double-stranded RNA (dsRNA) virus, have been detected directly in injured livers and hilar tissues or indirectly by the presence of serological markers of infection in patients with BA. In animal studies, when mice of age 2 days were infected with rotavirus, a member of Reoviridae, they developed cholestasis and biliary obstruction similar to that of human BA[4-6]. These findings suggest that infection with dsRNA viruses and following immune reactions may play an important role in the etiology of BA. However, the mechanisms by which dsRNA virus infection cause BA are still not known well.

Retinoic acid-inducible gene-I (RIG-I) is a member of cytoplasmic viral sensors, and recognizes viral dsRNA. RIG-I is expressed in biliary epithelial cells (BECs) and is involved in biliary innate immune reactions[7,8]. It is well known that activation of RIG-I signaling induces the expression of various cytokines and chemokines which induce inflammation by recruiting leukocytes.

In the experimental BA model mice, overexpression of various chemokines has been reported[9-12]. Among those chemokines, C-X-C motif chemokine 10 [CXCL10 (also known as IP-10; interferon gamma-induced protein 10)] was the only chemokine upregulated in all of above four studies. This suggests that CXCL10 may play an important role in the pathogenesis of experimental BA model animals. However, the role of CXCL10 in human BA is not fully elucidated.

To clarify the etiology and pathogenesis of human BA, we performed immunohistochemical study of RIG-I and CXCL10 in the surgically resected specimen. Furthermore, cultured HuCCT1 cells, a human cholangiocarcinoma cell line, were transfected with polyinosinic-polycytidylic acid [poly(I:C), a synthetic analog of viral dsRNA] and the role of RIG-I signaling in the expression of CXCL10 was examined.

Materials and methods

Human subjects

A total of 30 patients with BA (average age, 2.3 months; male/female, 13/17) were examined. Control subjects were 7 patients with hepatoblastoma (average age, 11 months; male/female, 4/3). These materials were surgically resected hilar tissues and hepatic tissues, which were retrieved from the files of the Department of Pathology and Molecular Medicine, Hirosaki University. Formalin-fixed, paraffin-embedded sections were prepared for histological observation and immunohistochemistry.

Reagents

RPMI-1640 medium, Lipofectamine RNAi MAX reagent and M-MLV reverse transcriptase were purchased from Invitrogen (Frederick, MD, USA). Poly(I:C) was from Sigma (St. Louis, MA, USA). SsoAdvenced SYBR Green DNA polymerase mix was from Bio-rad (Herculus, CA, USA). Oligonucleotide primers for PCR were custom-synthesized by Greiner Japan (Atsugi, Japan). An enzyme-linked immunosorbent assay (ELISA) kit for CXCL10 and an anti-CXCL10 antibody were obtained from R&D systems (Minneapolis, MN, USA). A non-silencing negative control siRNA and siRNAs against RIG-I were from Qiagen (Hilden, Germany). dNTP mix was from Thermo Fisher Scientific (Asheville, MA, USA). An anti-RIG-I antibody for immunohistochemical staining was from LifeSpan BioSciences (Seattle, WA, USA), and an anti-RIG-I antiserum for western blot analysis was prepared as described previously[13]. Anti-β-actin antibody was from GeneTex (Irvine, CA, USA).

Immunohistochemical staining

The surgically resected specimens were obtained from archives of pathology files maintained at the Department of Pathology and Molecular Medicine, Hirosaki University. Hilar tissues and liver tissues were procured from 30 patients with BA and non-inflamed hepatic tissues from 7 patients with hepatoblastoma were used as controls.

All specimens were fixed with 10% formalin and embedded in paraffin. The study conformed to the previsions of the Declaration of Helsinki and the ethical Declaration of the Japanese society of Pathology. Expression of RIG-I and CXCL10 in BECs were evaluated by immunohistochemical staining. Briefly, 4 μm-thick paraffin embedded sections of each specimen were deparaffinized, immersed in citrate buffer (pH 6.0) at 121℃ for 10 min for antigen retrieval. After blocking with normal rabbit serum, the specimens were incubated with a primary mouse anti-RIG-I antibody (1:100) or a goat anti-CXCL10 antibody (1:10) at 4℃ overnight. Finally, the sections were incubated with biotinylated anti-mouse IgG-IgA-IgM antibody or biotinylated anti-goat IgG antibody and the reaction was visualized using a Metal Enhanced DAB Substrate Kit, followed by light counterstaining with hematoxylin. We evaluated the degree of the expression for negative (−), weak positive (+), positive (++), and strong positive (+++) semiquantitatively. "Weak positive" is the case which was positive at high-power magnification (400 ×). "Positive" is the case which was positive at low-power magnification (100 ×). "Strong positive" is the case which was clearly stronger than positive cases.

Cell culture

HuCCT1 cells were cultured using RPMI-1640 medium supplemented with 10% fetal bovine serum (FBS) as described[14]. The cells were transfected with 0.4 - 50 ng/mL poly(I:C) and were incubated for up to 48h. In RNA interference experiments, the cells were transfected with siRNA against RIG-I or control siRNA and were incubated for 24h. Then the cells were transfected with poly(I:C) and were additionally incubated as indicated. The additional incubation time was decided according to pilot studies (data not shown).

Quantitative real-time reverse transcription (RT)-PCR analysis

Total RNA was extracted from the cells after the incubation as indicated. Subsequently, single-strand cDNA was synthesized using M-MLV reverse transcriptase. The expression levels of mRNAs for RIG-I, CXCL10 and GAPDH were examined with a quantitative real-time RT-PCR system. Values were normalized to GAPDH and expressed as relative fold increase. The primers used in this study are shown in Table 1.

Western blot analysis

In western blot analysis, the cells were lysed with Laemmli's reducing lysis buffer after incubation. The lysates were denatured and subjected to electrophoresis on a 7.5% polyacrylamide gel. The proteins on the polyacrylamide gel were transferred to a polyvinylidene difluoride membrane. The membrane was incubated with an anti-RIG-I antibody (1:10000) and anti-β-actin antibody (1:3000), followed by incubating with a secondary antibody labeled with horseradish peroxidase. A chemiluminescence substrate was used for immunodetection.

ELISA

After the incubation indicated above, the cell-conditioned medium was collected and centrifuged. The concentration of CXCL10 protein in the supernatant was quantified with an ELISA kit according to the manufacturer's protocol.

Table 1 Oligonucleotide primers for real-time quantitative RT-PCR

cDNA	primers
RIG-I	F: 5'- GCATATTGACTGGACGTGGCA -3'
	R: 5'- CAGTCATGGCTGCAGTTCTGTC -3'
CXCL10	F: 5'- ACCTCCAGTCTCAGCACCA -3'
	R: 5'- TGGGAGGATGGCAAATTCCATGGCA -3'
GAPDH	F: 5′ - CCACCCATGGCAAATTCCATGGCA -3′
	R: 5′ - TCTAGACGCCAGGTCAGGTCCACC -3′

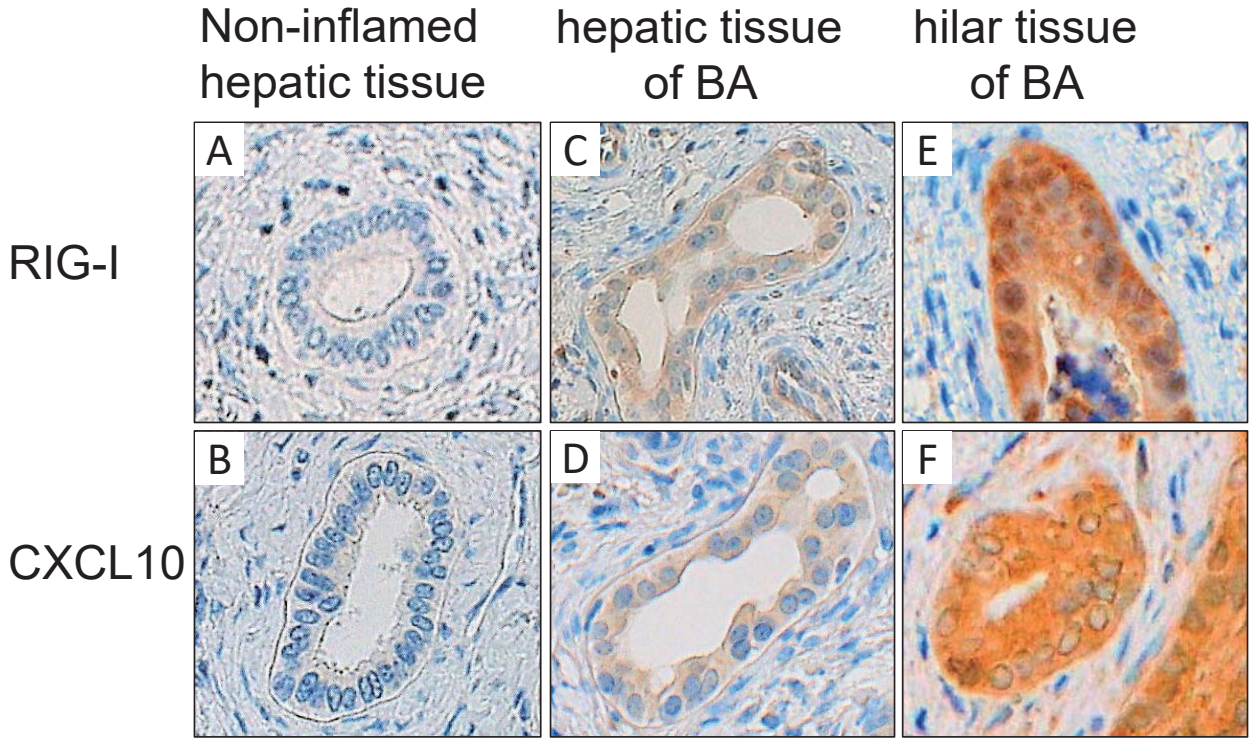

Figure 1 Expression of RIG-I and CXCL10 in biliary epithelial cells of BA. Immunohistochemical staining for RIG-I and CXCL10 was performed using non-inflamed part of hepatic tissues surgically resected from a patient with hepatoblastoma (A, B), and hepatic (C, D) and hilar tissues (E, F) from patients with BA. RIG-I and CXCL10 immunoactivity was detected in biliary epithelial cells in tissues from patients with BA, but not in most of control normal tissue. The expression was localized in cytoplasm of epithelial cells. In case of BA, the expression of RIG-I/CXCL10 in the hilar tissue was significantly stronger than in the hepatic tissue.

Statistics

The results of real-time RT-PCR and ELISA are expressed as mean ± SD (n=3). Data was analyzed by *t*-test and differences were considered significant at $P < 0.05$.

Ethical considerations

This study was approved by the ethics committees of Hirosaki University.

Results

RIG-I and CXCL10 are expressed in biliary epithelial cells of patients with BA

Immunohistochemical evaluation disclosed that expression of RIG-I and CXCL10 has been observed in cytoplasm of BECs to the various extent in patients with BA (C-1F). In contrast,

Table 2 Semiquantitative evaluation of the stainability of RIG-I

RIG-I staining		–	+	++	+++
control	Non-inflamed hepatic tissue	5	2	0	0
biliary atresia	Hilar tissue	1	1	11	13
	Hepatic tissue	2	11	17	0

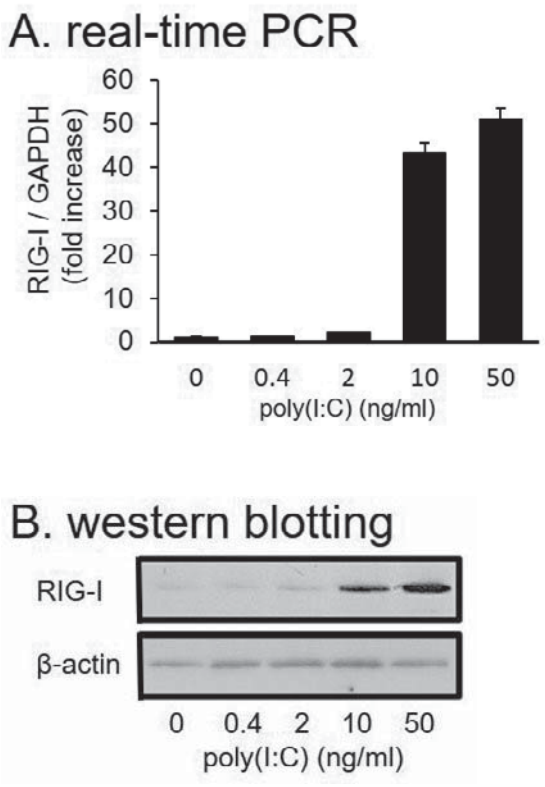

Figure 2 Polyinosinic-polycytidylic acid [poly(I:C)] induces the expression of RIG-I mRNA and protein in HuCCT1 human bile duct carcinoma cells in concentration-dependent manners. The cells were transfected with 0.4 - 50 ng/mL poly(I:C) and were cultured for 24 h. After the incubation, RNA was extracted from the cells. The expression of RIG-I mRNA was examined using quantitative real-time RT-PCR analysis (A). Values are shown as means+/–SD (n=3). The cells were lysed using Laemmli's reducing lysis buffer, and RIG-I protein in the cells was examined with western blotting (B).

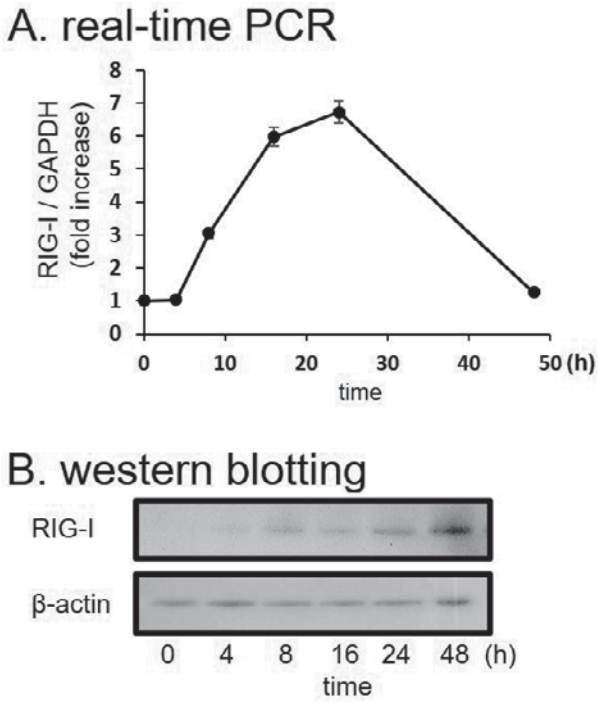

Figure 3 Poly(I:C) induces the expression of RIG-I mRNA and protein in HuCCT1 cells in time-dependent manners. The cells were transfected with 30 ng/mL poly(I:C) and were cultured for up to 48 h. Then the cells were subjected to quantitative real-time RT-PCR (A) and western blotting (B). Values in (A) are means+/–SD (n=3).

almost no RIG-I and CXCL10 expression has been found in controls (Fig. 1A, 1B). Marked expression of RIG-I and CXCL10 have been found in BECs of both hilar and hepatic tissue in BA. The expression of these molecules in hilar tissues has been stronger than in hepatic tissue. The strong positive has been observed only for hilar tissue of BA. On the other hand, in the hepatic tissue of BA, almost all cases were weak positive or positive (Table 2).

Poly(I:C) induces the expression of RIG-I in HuCCT1 human bile duct epithelial cells

Transfection of the cells with poly(I:C) has induced the expression of RIG-I mRNA and protein in concentration-dependent manners in

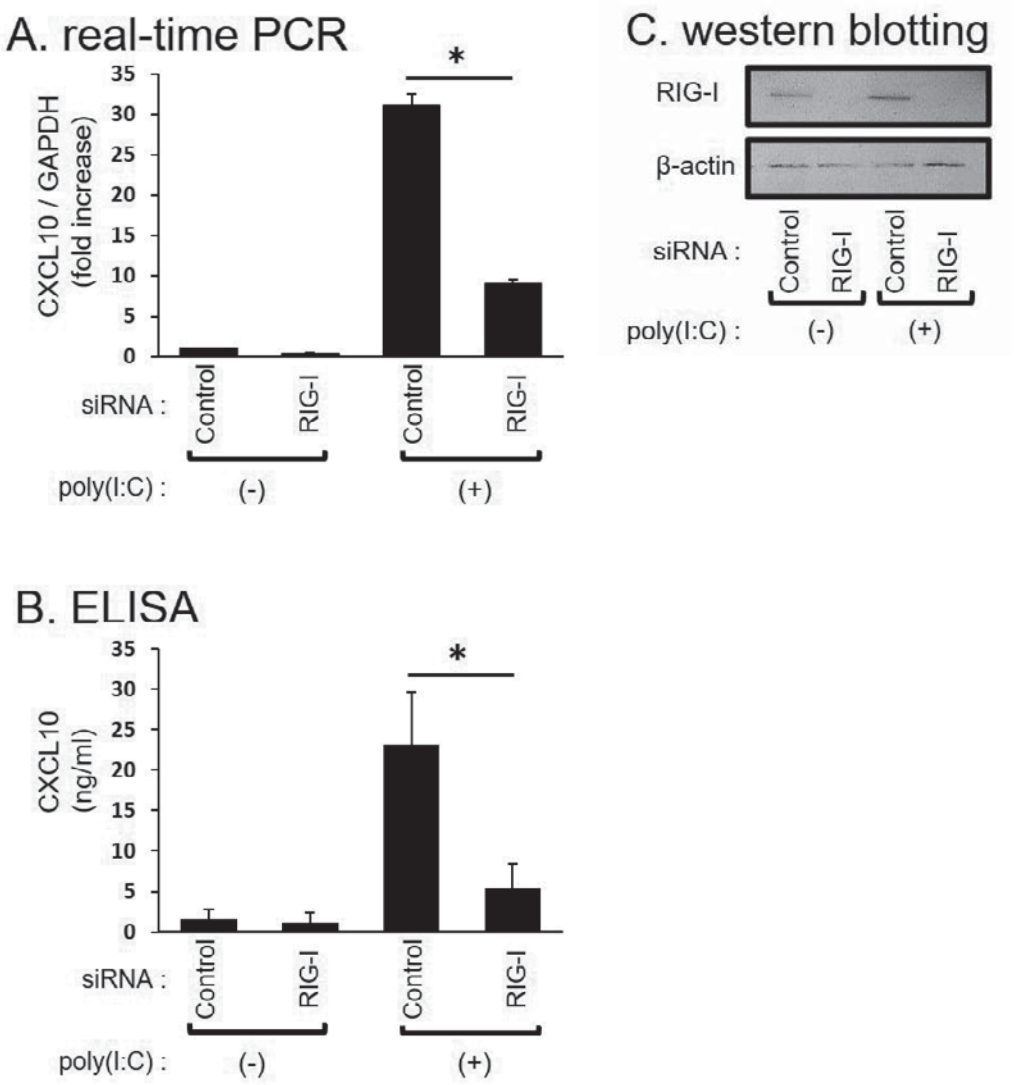

Figure 4 Knockdown of RIG-I inhibited the expression of CXCL10 in HuCCT1 cells transfected with poly(I:C). The cells were transfected with a specific siRNA against RIG-I or a control siRNA. After incubating for 24 h, the cells were transfected with 30 ng/mL poly(I:C). The cells were further incubated for 24 h. The cell-conditioned medium was collected and the cells were lysed. Expression of CXCL10 mRNA was quantified by real-time RT-PCR (A). The concentration of CXCL10 protein in the medium was measured by an ELISA (B) (n=3, *$p<0.05$). RIG-I protein expression was examined by western blotting (C).

HuCCT1 cells (Fig. 2A and B). RIG-I mRNA has began to increase 8 h after the transfection, and its expression reached a maximal level 24 h after the treatment (Fig. 3A). The increase of RIG-I protein has lagged behind the mRNA expression and significant increase has been detected 48h after the transfection with poly (I:C) (Fig. 3B).

RIG-I is involved in the expression of CXCL10 in HuCCT1 cells transfected with poly(I:C)

Transfection of HuCCT1 cells with a specific siRNA against RIG-I has inhibited the expression of CXCL10 mRNA and protein (Fig. 4A and B) induced by poly(I:C). Effective knockdown of RIG-I has been confirmed by western blotting (Fig. 4C).

Discussion

We have detected, in the present study, the expression of RIG-I and CXCL10 in BECs in tissues resected from BA patients. The expression in BECs of the hilar tissue has tended to be stronger than in BECs of the hepatic tissue. In addition, RIG-I mRNA and protein have been increased in HuCCT1 cells transfected with poly (I:C). We have found that knockdown of RIG-I inhibited the induction of CXCL10 in HuCCT1 cells transfected with poly(I:C), suggesting that activation of RIG-I and subsequent signaling is involved in poly(I:C)-induced CXCL10 expression.

Biliary tree is a duct to drain biles secreted by hepatocytes into duodenum. Recent studies have suggested that BECs play various roles associated with innate and acquired immunities in biliary tree in addition to the function as a draining duct of biles. Anatomically, biliary tree directly connects to duodenum, and duodenal microorganisms are believed to be a source of biliary bacterial infection[15]. To guard against bacterial invasion, BECs secrete mucus, polymeric immunoglobulin A, and antibiotics such as lactoferrin and defensin into bile[16-18]. Therefore, BECs can be regarded as a mucosal epithelium. Moreover, the biliary tract is anatomically divided into the intrahepatic and extrahepatic biliary tree, and the former is subdivided into intrahepatic large and small bile ducts[19]. Small bile ducts are further divided into the septal bile ducts, interlobular bile ducts, cholangiole, canal of Hering, and bile canaliculus according to their size. Extrahepatic bile ducts and intrahepatic large bile ducts are accompanied with peribiliary glands, and extrahepatic, intrahepatic large and septal bile ducts have mucous granules in supernuclear region[20]. Therefore, it is suggested that BECs of the central side, not peripheral side, are equipped with self-defense system. In our study, strong expression of RIG-I and CXCL10 in BECs of the hilar tissue have suggested that the viral infection with BECs of the central side may be important in the inflammatory reactions in BA.

RIG-I is innate immune-recognition receptor that recognize PAMPs. RIG-I contains an RNA helicase domain at C-terminus that functions as a sensor for dsRNA yielded by viruses, and two caspase recruitment domains (CARDs) at N-terminus that transmit the signaling to downstream[7]. In a former study, RIG-I is reported to be expressed and involved in CXCL10 expression in intestinal epithelial cells[21]. In cultured human BECs, RIG-I is shown to activate nuclear transcription factors including NF-kB and interferon regulatory factor-3 (IRF-3) and produce IFN-β by the introduction of poly(I:C)[8,22]. NF-kB is a key molecule in the expression of various inflammatory genes and IRF-3 is an important transcriptional factor in antiviral innate immune reaction. The results of present study suggest that RIG-I may be involved in the CXCL10 expression in human BECs of BA.

Although the mechanisms by which CXCL10 contribute to the etiology of BA has not been clarified yet in the present study. CXCL10 may induce the chemotaxis of leukocytes and following inflammation. In addition, it has been suggested that CXCL10 is a profibrotic factor which participates in a crosstalk between hepatocytes, hepatic stellate cells, and immune cells[23]. Even after Kasai portoenterostomy, fibrosis of the liver progresses in about 70% of children. Therefore, CXCL10 may play an important role in the hepatic fibrogenesis of BA patients. The detailed role of RIG-I-CXCL10 cascade in fibrosis following inflammation should be further investigated.

In summary, the present study has suggested that this cascade may contribute to the dsRNA virus-induced inflammatory reactions in the BECs, and may be involved in the etiology of human BA.

Conflicts of interest statement

All authors have no conflict of interest directly relevant to the content of this article.

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