

Original paper

# The usefulness of immunohistochemical staining of bile tracts in biliary atresia

Joanna Cielecka-Kuszyk<sup>1</sup>, Maria Janowska<sup>2</sup>, Małgorzata Markiewicz<sup>2</sup>, Piotr Czubkowski<sup>3</sup>, Anna Ostoja-Chyżyńska<sup>2</sup>, Joanna Beata Bierła<sup>1</sup>, Bożena Cukrowska<sup>1</sup>, Joanna Pawłowska<sup>3</sup>

<sup>1</sup>Department of Pathology, The Children's Memorial Health Institute, Warsaw, Poland

<sup>2</sup>Department of Pediatric Surgery and Organ Transplantation, The Children's Memorial Health Institute, Warsaw, Poland

<sup>3</sup>Department of Gastroenterology, Hepatology, Nutritional Disturbances and Pediatrics, The Children's Memorial Health Institute, Warsaw, Poland

## Abstract

**Aim of the study:** To assess ductular proliferation (DP) and ductal plate malformation (DPM) in biliary atresia (BA) by means of immunohistochemical staining using cytokeratins CK7 and CK19 and neural cell adhesion molecule (NCAM) antibody CD56.

**Material and methods:** In 10 cases of BA, liver surgical biopsies obtained at the time of hepatoportoenterostomy were stained with H&E, PAS, Gomori and Azan methods. Immunohistochemical technique was used to outline bile ducts, ductular reaction, reactive bile duct/ductules and DPM by CK7, CK19 and NCAM antibody CD56.

**Results:** We found fibrosis, bile stasis and mild inflammation in all cases. In the routine staining DP was not seen in 3 cases. The immunohistochemical staining by means of CK19 was helpful in the detection of DP, and allowed it to be demonstrated in all cases. The biliary epithelial cell markers for CD56, CK7, CK19 were used for demonstration of bile duct cell but not hepatocyte alterations in the structure of intrahepatic biliary ducts and different stages of maturation. CD56 as a marker of immature bile ducts was expressed on biliary epithelium of bile ducts and bizarre forms of DPM in 6 cases. The positive expression of CD56 corresponded to the co-localization of CK19 of DPM, but not CK7, to the ductular reaction at the limiting plate of portal tracts. CD7, considered as a marker of DP, also stained ductal hepatocytes and multipotential oval cells, and was a marker of DPM in 3 cases.

**Conclusions:** Use of CK7, CK19 and CD56 is helpful in BA diagnosis and allows differentiation of the stage of developing bile duct cells according to the expression pattern.

**Key words:** biliary atresia, liver biopsy, immunohistochemistry.

## Address for correspondence:

Joanna Cielecka-Kuszyk, Department of Pathology, The Children's Memorial Health Institute, Warsaw, Poland,  
e-mail: joannack@onet.pl

## Introduction

Biliary atresia (BA), even though a rare disease, is the main cause of neonatal cholestasis [1]. The nature of the disease is the obstruction or enabling the outflow of bile from the liver due to progressive inflammation, fibrosis, and hypertrophy of the extra- and intrahepatic bile ducts. The clinical picture corresponds to the progressive damage to the biliary tract with symptoms of cholestasis from the first weeks of life. Most often, be-

tween 2 and 4 weeks of life, a child is diagnosed with jaundice, acholic stools, dark urine, and hepatomegaly. However, most of the time, children who were born with normal weight develop properly in the early stages of the disease. Symptoms appear after the jaundice-free period. In this type of BA, called isolated form, congenital anomalies are not observed. The second (fetal) type is associated with the biliary atresia splenic malformation (BASM) syndrome [2]. In this case, the onset of symptoms occurs by three weeks of age. These can

include splenic malformation (usually polysplenia, but also asplenia and double spleen), cardiac anomalies, disorders of visceral symmetry (e.g. situs inversus and malrotation), and malformations of the intra-abdominal veins (e.g. absent inferior vena cava, preduodenal portal vein) [3]. The pathogenesis of the disease is still unclear. It is a multi-factorial disease; factors include genetic predisposition with glypican 1 (GPC1), adducin 3 (ADD3) and ADP-ribosylation factor 6 (ARF6), genes important in embryonic development and organogenesis, such as a potential BA susceptibility gene [4], environmental factors such as biliary toxin bilitresone [5], infectious causes including reovirus, rotavirus, cytomegalovirus (CMV) and Epstein-Barr virus (EBV) [6, 7], and immune dysregulation, e.g. decreased Treg subset of CD4+ function [8]. Still, BA requires urgent diagnosis due to the necessity to qualify patients for surgical treatment before the age of 8 weeks. The final diagnosis is based on an intraoperative evaluation of the biliary tract and cholangiography, but liver biopsy is a helpful tool for evaluation of microscopical changes in the liver, especially ductular proliferation (DP), ductal plate malformation (DPM), fibrosis and cholestasis. However, these features, especially DP, and DPM, are not always distinct using routine staining. Visualization of cytokeratins and neural cell adhesion molecules in the immature ductal cells by means of the immunohistochemical method can be a useful tool for the microscopic examination of the immature biliary structures in the liver. The aim of the study is to assess the value of immunohistochemical staining in the diagnosis of BA.

## Material and methods

In 10 cases of BA (6 males and 4 females) liver fragments consisting of surgical wedge biopsies were

obtained at the time of Kasai hepatopertoenterostomy (Table 1). The age of the children at the time of the Kasai procedure ranged from 24 to 101 days (mean 64 days). The laboratory results were presented as total bilirubin (mean 8.48 mg/dl), direct bilirubin (mean 7.36 mg/dl), prothrombin time/international normalized ratio (INR) (mean 1.13), alanine aminotransferase (ALT) (mean 148 IU/l), aspartate aminotransferase (AST) (mean 188 IU/l),  $\gamma$ -glutamyl-transpeptidase (GGTP) (mean 608 IU/l) (Table 1).

All 10 surgical biopsy specimens were fixed in buffered formalin and embedded in paraffin blocks. In each case 4 micrometer thick sections, displaying at least 10 portal spaces, were routinely stained with hematoxylin and eosin, the periodic acid-Schiff method with and without diastase, Gomori silver impregnation and Azan stain. Histological interpretation was performed using internationally accepted criteria [9] and all histopathological features were finally scored using the four-degree scale for the grade (inflammatory activity) and stage (fibrosis) of the disease. Additionally inflammatory infiltrates around bile ducts, giant cell transformation of hepatocytes, proliferating ductules, DPM in the form of circumferential biliary structures within the connective tissue of portal tracts resembling primitive ductal structures, portal/acinar bile plugs and hepatocyte rosette formations were examined and categorized from absent (-), mild (+) to severe (++) . Ductular reaction/proliferation was defined as the presence of increased ductular profiles without a lumen at the limiting plate or interface, often detected only by means of immunohistochemical methods. Portal stromal edema was observed as clearing and separation of the extracellular matrix resulting in expansion of the portal tract.

**Table 1.** Baseline clinical and laboratory characteristics of patients with biliary atresia

Patient no.	Sex	Age at birth (hbd)	Age at Kasai procedure (days)	ALT (IU/l)	AST (IU/l)	GGTP (IU/l)	Total bilirubin (mg/dl)	Direct bilirubin (mg/dl)	INR
1. GH	F	39	87	145	264	514	6.58	6.00	1.03
2. BR	F	36	35	126	176	504	11.11	10.27	1.14
3. KM	F	38	101	176	228	1652	9.46	8.27	1.08
4. MD	F	39	71	174	202	467	15.53	12.88	1.08
5. FK	M	39	91	153	232	583	7.49	6.12	1.66
6. SA	M	38	56	342	308	90	5.30	4.72	1.15
7. PA	M	38	65	86	110	759	7.68	7.46	1.19
8. BN	F	39	24	70	66	689	4.55	3.56	0.88
9. SzA	M	41	62	100	152	587	8.39	7.78	0.99
10. SzP	M	39	50	112	146	244	8.74	6.62	1.11

Unstained slides were also examined by means of immunohistochemistry using mouse anti-human antibodies (Dako) for cytokeratins CK 7, CK19 and neural cell adhesion molecule antibody CD56 to outline bile ducts and ductular reaction, reactive bile duct/ductules and DPM. Endogenous peroxidase was quenched and non-specific signals were blocked by addition of 0.1% bovine serum albumin. The antibodies (1 : 50-1 : 200) were incubated at 37°C overnight. The signals were revealed by the Dako EnVision System. The control group consisted of 4 surgical biopsies obtained during an operation from children with choledochal cysts and all specimens were stained with the same methods as the examined group (Table 2).

## Results

The histopathological diagnosis of BA was based on the presence of portal/bridging fibrosis, DP, presence of DPM, cholestasis, giant cell transformation and rosette formation of hepatocytes (Table 2). In all samples liver fibrosis as a characteristic morphological feature for BA was described as periportal fibrosis (2 patients with mean age 37 days at Kasai procedure), bridging fibrosis moderate to severe stage (6 patients with mean age 69 days at Kasai procedure) and cirrhosis (2 patients with mean age 76 days at Ka-

sai procedure). These changes were accompanied by mild inflammatory infiltrates composed of lymphocytes and rare granulocytes (8 patients with minimal and 2 patients with mild inflammation according to Batts and Ludwig scale). In 5 cases, inflammatory infiltrates were found in the wall of bile ducts/ductules, but in 4 cases inflammatory cells were also distributed in the fibrotic tissue between portal tracts.

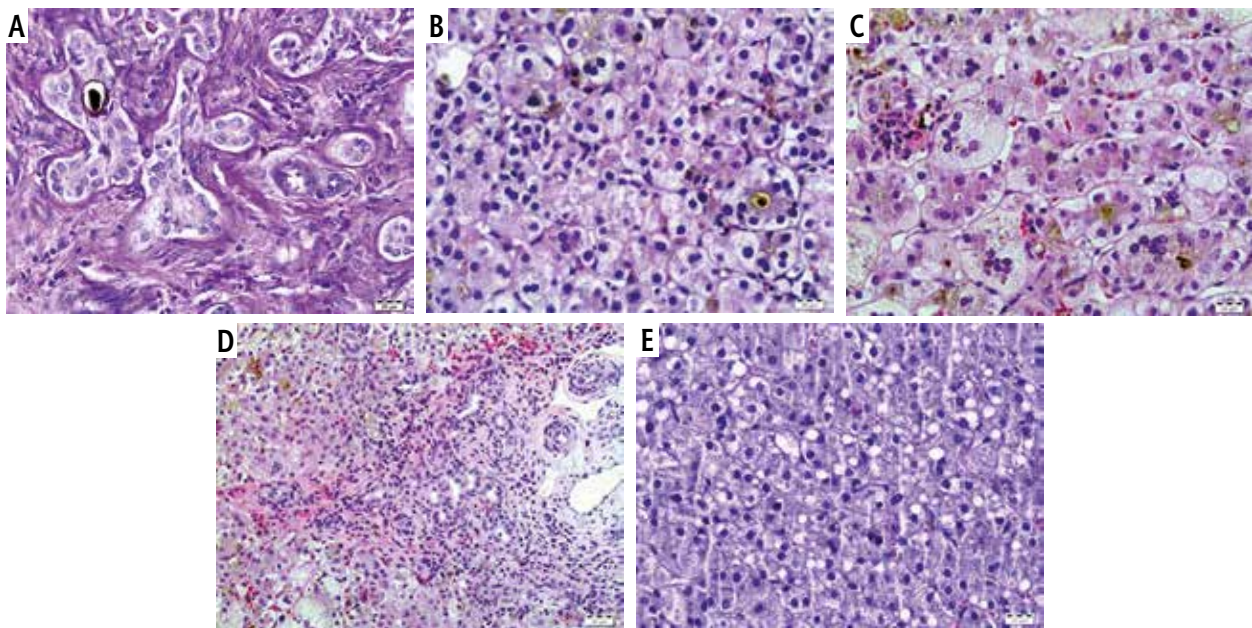
Bile plugs were seen in every case and were accompanied by stromal edema. Accumulation of bile plugs, as a hallmark of BA, was found in bile ducts, DPM structures in the portal tracts and inside acinar rosette formations. However, intralobular distribution of bile plugs was less frequent than those in portal tracts. Giant cell transformation and lobular extramedullary hematopoiesis were observed in 6 cases and were associated with the presence of interlobular bile plugs and rosette formations of the liver cells. We found steatosis of 30% of hepatocytes in one case (Fig. 1).

In the routine staining (H&E, PAS, Azan, Gomori silver impregnation) DP was not seen in 3 cases. The immunohistochemical staining by means of CK19 was helpful in the detection of DP, categorized as increased ductular profiles at the limiting plate or interface in generalized distribution, and allowed this feature to be demonstrated in all 10 cases. The biliary epithelial cell markers for CD56, CK7, CK19 were used for demon-

**Table 2.** Histopathological and immunohistochemical characteristics of patients with biliary atresia

Patient no.	G/S	Ductitis/ductal proliferation	Giant cell transformation	DP			DPM			Bile plugs	Rosette formation
				CK7	CK19	CD56	CK7	CK19	CD56		
1. GH 3854/18	1/3	+/+	-	+	+	-	+	+	-	+	-
2. BR 508/20	1/3	+/+	-	-	+	-	-	+	+	+	-
3. KM 6926/17	1/3	-/-	++	+	+	-	-	-	-	+	+
4. MD 4290/17	1/3	-/-	++	+	+	-	-	+	+	+	+
5. FK 8150/17	1/4	+/+	+	+	+	-	-	-	-	+	-
6. SA 3551/19	1/3	-/-	++	+	+	-	+	+	-	++	++
7. PA 6069/19	1/3	+/++	+	+	+	-	-	+	-	++	+
8. BN 3078/19	1/2	-/+	- steatosis 30%	-	+	-	-	-	-	++	++
9. SzA 2686/19	2/4	-/+	-	+	+	-	-	-	-	+	-
10. SzP 8433/17	2/2	+/+	++	-	+	-	+	+	+	++	+

DP – ductular proliferation, DPM – ductal plate malformation



**Fig. 1.** Histopathological changes in BA. **A)** Intraductal bile plug H&E. **B)** Intralobular bile plug with rosette formation H&E. **C)** Giant cell transformation of hepatocytes, surgical hepatitis H&E. **D)** Ductular and ductal proliferation in the portal space. **E)** Macrovesicular steatosis of hepatocytes

stration of bile duct cell but not hepatocyte alterations in the structure of intrahepatic biliary ducts and different stages of maturation. CD56 as a marker of immature bile ducts was expressed on biliary epithelium of bile ducts and bizarre forms of DPM in 6 cases. The positive expression of CD56 corresponded to the co-localization of CK19 of DPM, but not CK7 to the ductular reaction at the limiting plate of portal tracts. CD7 was usually considered as a marker of DP, but also stained ductal hepatocytes and multipotential oval cells and in 3 cases was a marker of DPM. This DP was not always seen in routine staining with H&E, PAS or Azan methods but the application of immunohistochemistry allowed it to be detected in every examined case: the expression of CK7 was present in 7 examined biopsies, and CK19 in all cases (Fig. 2). The expression of CD56 was usually absent in the proliferating bile ductules.

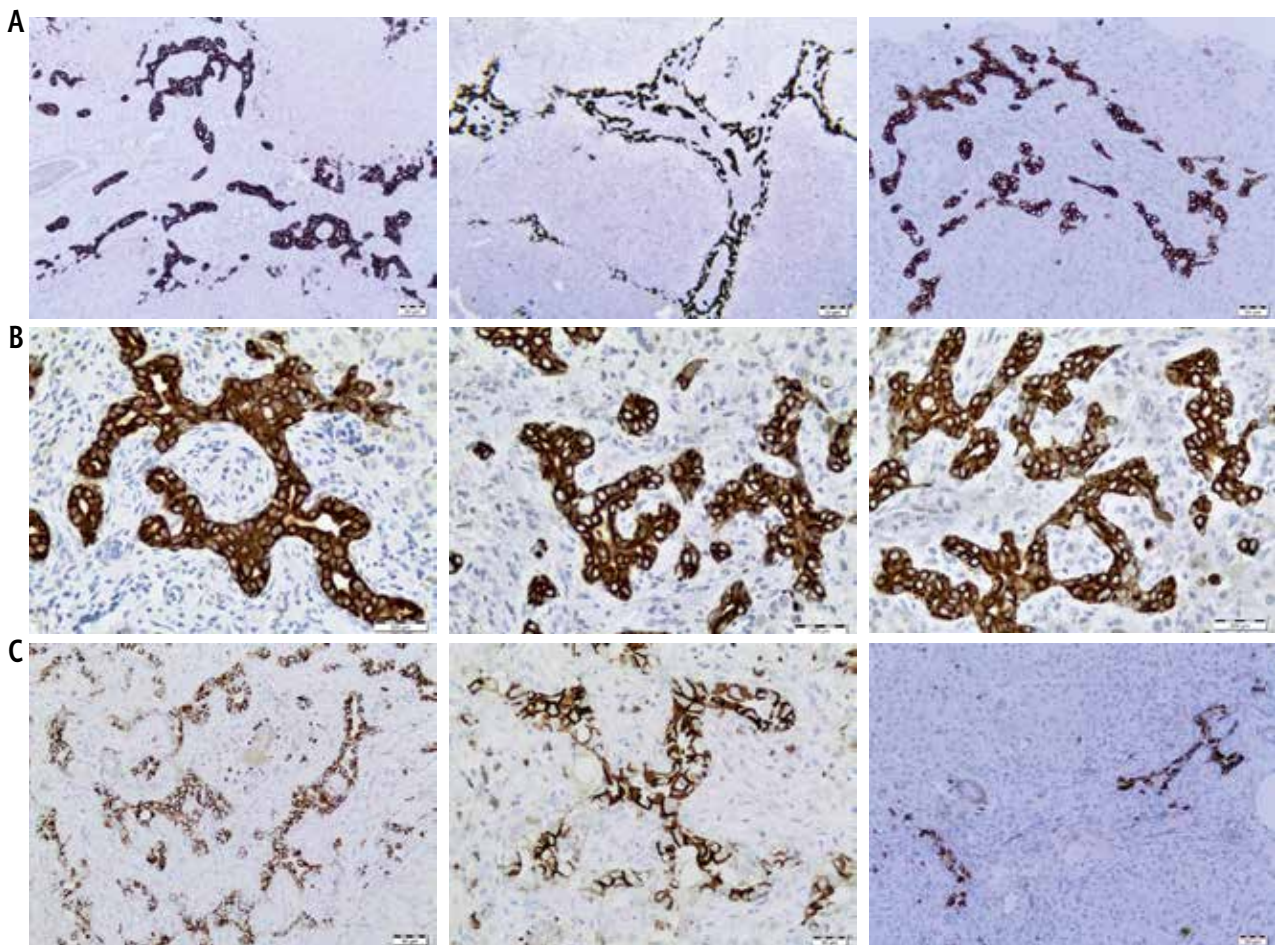
Ductal plate malformation was present in 6 and absent in 4 cases independently of the degree of fibrosis. Bizarre morphology of DPM was associated with a central fibrotic core and small central vessel, similar to that observed during normal bile duct development in the fetal liver. DPM centrally located in the portal tracts, not always seen in routine staining, was associated with bile plugs but not with ductal or DP in the portal tracts. DPM showed expression of CD56 (4 cases), CK7 (3 cases) and CK19 (6 cases).

In the control group we found bile ducts and mild DP positive for CK19 and CK7. The immunostaining for CD56 was positive in the wall of blood vessels, singular cells in the bile ducts and along acinar sinusoids.

## Discussion

The interpretation of liver biopsies in BA is challenging according to the histological features based on bile plugs in portal bile ducts/ductules, portal stromal edema, higher stages of portal fibrosis and characteristic DP. These features, together with the presence of DPM, are helpful in the differentiation between BA and nonBA cholestasis. The presence of DP and DPM is not always obvious by routine staining and in our material has been shown as different, bizarre forms stained by CK7, CK19 and CD56. Microscopic pictures of the histological changes in BA present similar features which occur in the embryonic development when the ductal plates are remodeled into mature tubular ducts. In BA lack of remodeling results in DPM. DPM is found in approximately 20% of BA cases and its presence signifies an adverse outcome [10]. We did not find any DPM structures in 4/10 cases (40%) but it did not influence the prognosis of BA [11]. However, the immunohistochemical reactions by means of cytokeratins and neural cell adhesion molecule antibodies to outline immature bile structures are very helpful for the diagnosis of BA. Ductular proliferation is suggested to be associated with liver regeneration in the state of liver fibrosis. The periportal ductular reaction occurs during hepatic regeneration in response to injury. The acute stage of hepatic regeneration is characterized by the proliferation of ducts, and during the chronic stage there is a proliferative and metaplastic transformation of hepatocytes into metaplastic bile





**Fig. 2.** Immunohistochemical presentation of ductular reaction and ductal plate malformation by immunohistochemical staining CK7 (A), CK19 (B), CD56 (C)

ducts [12]. This DP can be demonstrated by means of CK7, which reacts with biliary epithelial cells but also with progenitor hepatocytes. The reaction with CD56 allows one to identify immature biliary epithelial cells and to determine that in the presence of DPM. Biliary epithelial cells expressing CD56 positivity are also present in higher numbers compared to those with neonatal hepatitis and choledochal cysts. The presence of CK7 together with CD56 positivity can be a sign of a newly formed bile structure and often shows no lumen, suggesting inhibition of the maturation process in BA. As the developing intrahepatic bile ducts are classified into the ductal-plate stage, remodeling stage (migration of biliary cells from the ductal plate into the portal mesenchyme) and remodeled stage (mature bile ducts), the expression of CK7 begins at the remodeling stage and is characteristic for the remodeled stage (absent in the ductal-plate stage and weak in the remodeling stage), CK19 occurs in all three stages: ductal plate, remodeling and remodeled stage. CD56 is characteristic for the remodeling stage [13]. Application of two different cytokeratins – CK7 and CK19 –

and CD56 allowed us to speculate that the maturation arrest was established in 3 cases at ductal-plate/early remodeling stage (CK19 positivity), and 3 other cases at the remodeling/remodeled stage (CD56, CK7, CK19 positivity). The use of routine staining in BA does not allow one to determine the possible stage of the liver injury, or the exact morphology of DPM. The presence of CD56 also implies the progression of BA to liver fibrosis [14]. Presence of DPM shows the potential sequence of the prenatal bile duct injury by congenital infection, environmental toxins or genetic susceptibility in BA and has been associated with a worse clinical outcome [15, 16]. Some authors did not find any histological features that predicted successful bile drainage after Kasai portoenterostomy, apart from severity of portal fibrosis. DPM and bile duct injury were associated with shorter survival with the native liver [17, 18]. Most cases of BA (80%) are considered perinatal or acquired, while a minority (20%) are congenital and are expected to lead to DPM [6]. However, many authors report a worse outcome with increasing fibrosis, lobular inflammation, biliary proliferation and DPM

[16, 19]. Our earlier report indicated that the severity of fibrosis corresponded neither with the age at hepatoportoenterostomy nor with the laboratory findings before the operation but increased the risk of portal hypertension [11].

The detection of DP and DPM by means of CK7, CK19 and CD56 is important not only for the diagnosis but also for the risk of early cirrhosis. Our selected group of 10 patients did not show any difference in liver fibrosis according to age, but the youngest patient, aged 24 days at hepatoportoenterostomy, had only periportal fibrosis and fibrous portal to portal septa without cirrhotic changes but severe bile stasis.

Differential diagnosis of BA with other forms of infantile cholestasis such as neonatal hepatitis, indeterminate cholestasis, and Alagille syndrome is often difficult because of similar histopathological changes occurring in the neonatal life. Immunohistochemical positivity for CD56 is also helpful in the differential diagnosis because of the expression on immature bile ducts in BA, but not in patients with neonatal hepatitis.

## Conclusions

Ductular proliferation at the periportal zone and bizarre forms of DPM discovered by immunohistochemical methods by use of CK7, CK19 and CD56 are helpful in the diagnosis of BA and make it possible to differentiate the stage of developing bile duct cells according to the expression pattern.

## Disclosure

The authors declare no conflict of interest.

## References

1. Verkade HJ, Bezerra JA, Davenport M, et al. Biliary atresia and other cholestatic childhood diseases: advances and future challenges. *J Hepatol* 2016; 65: 631-642.
2. Davenport M, Savage M, Mowat AP, et al. Biliary atresia splenic malformation syndromes: an etiologic and prognostic subgroup. *Surgery* 1993; 113: 662-668.
3. Lakshminarayanan B, Davenport M. Biliary atresia: a comprehensive review. *J Autoimmun* 2016; 73: 1-9.
4. Tsai EA, Grochowski CM, Loomes KM, et al. Heterozygous deletion of FOXA2 segregates with disease in a family with heterotaxy panhypopituitarism and biliary atresia. *Hum Mutat* 2015; 36: 631-637.
5. Lorent K, Gong W, Koo KA, et al. Identification of a plantisoflavonoid that causes biliary atresia. *Sci Transl Med* 2015; 7: 286-297.
6. Averbukh LD, Wu GY. Evidence for viral induction of biliary atresia. *J Clin Transl Hepatol* 2018; 6: 410-419.
7. Kobayashi H, Stringer MD. Biliary atresia. *Semin Neonatol* 2003; 8: 383-391.
8. Bezerra JA, Wells RG, Mack CL, et al. Biliary atresia: clinical and research challenges for the twenty-first century. *Hepatol* 2018; 68: 1163-1173.
9. Batts K, Ludwig J. Chronic hepatitis. An update on terminology and reporting. *Am J Surg Pathol* 1995; 19: 1409-1417.
10. Pacheco MC, Campbell KM, Bove KE. Ductal plate malformation like arrays in early explants after Kasai procedure are independent of splenic malformation complex (heterotaxy). *Pediatr Dev Pathol* 2009; 12: 355-360.
11. Czubkowski P, Cielecka-Kuszyk J, Rurarz M, et al. The limited prognostic value of liver histology in children with biliary atresia. *Ann Hepatol* 2015; 14: 902-909.
12. Cocjin J, Rosenthal P, Bulson V, et al. Bile ductule formation in fetal, neonatal, and infant livers compared with extrahepatic biliary atresia. *Hepatol* 1996; 24: 568-574.
13. Sasaki H, Nio M, Iwami D, et al. Cytokeratin subtypes in biliary atresia: immunohistochemical study. *Pathol Int* 2001; 51: 511-518.
14. Zhang RZ, Yu JK, Peng J, et al. Role of CD56-expressing immature biliary epithelial cells in biliary atresia. *World J Gastroenterol* 2016; 22: 2545-2557.
15. Santos JL, Kieling CO, Meurer L, et al. The extent of biliary proliferation in liver biopsies from patients with biliary atresia at portoenterostomy is associated with the postoperative prognosis. *J Pediatr Surg* 2009; 44: 695-701.
16. Shimadera S, Iwai N, Deguhi H, et al. Significance of ductal plate malformation in the postoperative clinical course of biliary atresia. *J Pediatr Surg* 2008; 43: 304-307.
17. Ferreira AR, Queiroz TC, Vidigal PV, et al. Multivariate analysis of biliary flow-related factors and post-Kasai survival in biliary atresia patients. *Ar Gastroenterol* 2019; 56: 71-78.
18. Russo P, Magee JC, Anders RA, et al. Key histopathological features of liver biopsies that distinguish biliary atresia from other causes of infantile cholestasis and their correlation with outcome: a multicenter study. *Am J Surg Pathol* 2016; 40: 1601-1615.
19. Lampela H, Kosola S, Heikkilä P, et al. Native liver histology after successful portoenterostomy in biliary atresia. *J Clin Gastroenterol* 2014; 48: 721-728.