



Title	Apelin is a marker of the progression of liver fibrosis and portal hypertension in patients with biliary atresia
Author(s)	Chen, Wei; Oue, Takaharu; Ueno, Takehisa et al.
Citation	Pediatric Surgery International. 2013, 29, p. 79-85
Version Type	AM
URL	<a href="https://hdl.handle.net/11094/99832">https://hdl.handle.net/11094/99832</a>
rights	© 2012 Springer-Verlag Berlin Heidelberg
Note	

*The University of Osaka Institutional Knowledge Archive : OUKA*

<https://ir.library.osaka-u.ac.jp/>

The University of Osaka

# **Apelin is a marker of the progression of liver fibrosis and portal hypertension in patients with biliary atresia**

## **Abstract**

### **Background/Aim**

Apelin, the endogenous ligand of the angiotensin-like-receptor 1 (APJ), has recently been suggested to play an important role in liver disease. This study investigated the apelin expression in different stages of biliary atresia (BA) and investigated whether it is associated with the progression of liver fibrosis and portal hypertension in the patients with BA.

### **Methods**

Liver tissues were obtained from patients at an early-stage of BA at the time of Kasai's procedure (KP; n = 4), the follow-up stage of BA after KP (Post-KP; n = 29), the end-stage of BA at the time of liver transplantation (LT; n = 9), and the follow-up stage of BA after LT (Post-LT; n = 30). Normal liver samples from children with choledochal cysts and hepatoblastoma served as controls (CO; n = 5). Real-time quantitative reverse transcriptase polymerase chain reaction (QRT-PCR) for apelin mRNA expression and immunohistochemistry for apelin and its receptor (APJ) expression were conducted. The relationship between the mRNA expression of apelin and the liver fibrosis stage (New Inuyama classification) and clinical presentation were analyzed.

### **Results**

The apelin mRNA expression level was significantly higher in the LT group than in the CO ( $p < .05$ ), KP ( $p < .01$ ), Post-KP group ( $p < .001$ ) and Post-LT group ( $p < .001$ ). The analysis of apelin mRNA expression during the progression of BA in the same patients showed that mRNA levels were increased nearly 10-fold in LT in comparison to their previous stages (i.e. KP and Post-KP). The apelin mRNA expression level was significantly higher in cirrhotic liver (stage 4, New Inuyama classification) than in fibrotic liver (stage 4 versus stage 0-1,  $p < .05$ ; stage 4 versus stage 2-3,  $p < .01$ ). Significant linear correlations were observed between the apelin mRNA level and serum total bilirubin ( $r_s = 0.520$ ,  $p < .001$ ) and the grade of esophageal

varices ( $r_s = 0.522$ ,  $p < 0.01$ ). The immunohistochemical study revealed that in control liver tissue, apelin was mainly localized in the perivenular areas (large portal vein and ventral vein), and slightly detected in the sinusoid and parenchyma; in KP and Post-KP liver tissue, apelin was observed mainly in perivenular areas and capillaries and moderately in the sinusoids; in LT liver tissue, intense apelin immunoreactivity was detected mainly in perivenular, sinusoidal and parenchyma areas; whereas APJ APJ was almost undetected in perivenular areas in all stage of BA.

## **Conclusion**

The apelin expression level accurately reflects the severity of hepatic fibrosis and esophageal varices and therefore could be used as a prognostic factor to estimate the timing of liver transplantation in BA patients.

**Keywords:** apelin, biliary atresia, liver fibrosis, esophageal varices, Kasai's procedure, liver transplantation.

## **Introduction**

Apelin, initially isolated by Tatemoto [1] and his coworkers from bovine stomach homogenates in 1998, has been recognized as the endogenous ligand of angiotensin-like-receptor 1 (APJ), human orphan G-protein-coupled receptor, which has a close identity with the angiotensin II receptor, but does not bind angiotensin-II [2].

Apelin and its receptor are highly expressed in the central nervous system and in peripheral tissues, where it is involved in the regulation of the cardiovascular tone [3], cardiac contractility [4], glucose metabolism [5], gastrointestinal track physiology [6], and water homeostasis [7].

Recent studies have demonstrated that apelin is overexpressed in hepatic stellate cells (HSCs) of both cirrhotic human and rats [8] [9] and enhanced expressions on proliferative hepatic arterial capillaries in human cirrhotic liver [10].

Biliary atresia (BA) is characterized by complete obliteration of extra hepatic bile duct. Although bile flow can be established by the Kasai portotenterostomy (KP), progressive liver fibrosis and portal hypertension continue to develop in postoperative patient with BA [11]. Therefore, apelin maybe over-express in the liver of BA patients.

However, to date, there is no information available regarding the apelin expression in liver tissue of BA patients, nor relationship between apelin expression and progression of liver fibrosis and portal hypertension in patients with BA. The present study was undertaken to investigate the apelin mRNA expression in different stages of BA patients, and especially focus on the correlation between its expression and clinical features such as liver function, hepatic fibrosis and esophageal varices.

## Materials and methods

### *Samples and Patients*

55 BA patients including 19 boys and 36 girls with mean age  $8.4 \pm 8.2$  years (rang, 37 days - 29.6 years) who were treated and followed at Osaka University Hospital between June 2009 and June 2012 were included in this study. Total 73 liver tissues were taken from them, including 4 wedge needle biopsy tissues taken at the time of KP, 29 needle biopsy samples from Post-KP patients, 9 liver tissues taken at time of LT, and 30 needle biopsy samples from Post-LT patients. Control liver samples included nontumor parts of surgical removed liver tissues from 3 children with hepatoblastoma and 2 near-normal tissues from patients with choledochal cyst. All liver tissues in our study were obtained after acquiring written informed consent from parents or healthy adult donors. The protocol for our study was approved by the Ethics Committee of the Institutional Review Board of Osaka University Hospital.

### *Evaluation of apelin mRNA expression using quantitative real-time PCR.*

Total RNA was extracted from frozen sample using TRIzol RNA isolation reagent (Life Technologies, Tokyo, Japan) according to the manufacturer's recommendations. Template cDNA was obtained by reverse transcription of 1µg of total RNA using a cDNA synthesis Kit (Prime Script™ RT-PCR Kit, TaKaRa, Japan). The reaction mixtures were incubated at 30°C for 10 min, 42°C for 30 min and 95°C for 5 min. The cDNA was diluted 5-fold for real-time PCR. The sequence of the primers in this study was following: sense primer 5'-GGCCATCACCAGCCATTCCTTG -3' and antisense primer 5'-GGGCATCAGGCTCTTGTCTTCTCT -3'. The quantification of gene-expression levels for apelin was carried out by real-time quantitative PCR on an ABI ViiA™ 7 System (TaqMan,

Perkin-Elmer Applied Biosystems). SYBR Premix Ex Taq™ II kit (TaKaRa) was used for real-time monitoring of amplification (45 cycles: 95°C/15 s, 60°C/1 min). We used the comparative cycle threshold (Ct) method to calculate relative mRNA expression. All quantifications were normalized by the corresponding expression of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA expression (forward primer: 5'-GAAGGTGAAGGTCGGAGTCA -3'; reverse primer: 5'-GAAGATGGTGATGGGATTTC -3').

#### *Immunohistochemistry*

Liver tissues were fixed in formalin and embedded in paraffin. From the paraffin blocks, 4 µm sections were cut, deparaffinized with hemo-de, and dehydrated using graded ethanol. They were incubated overnight at 4°C with 1:800 dilution of anti-apelin rabbit antibody (Phoenix Pharmaceuticals, INC., Burlingame, CA • U.S.A.) and 1:500 dilution of apelin receptor rabbit antibody (MBL International, Woburn, MA, USA). After washing with Dako Wash Buffer (Dako, Tokyo, Japan), the sections were incubated with peroxidase labeled polymer conjugate (Envision<sup>®</sup> system) (Dako, Tokyo, Japan) at room temperature for 30 min and then reacted with DAB chromogen. The sections were finally counter stained with hematoxylin for light microscopic study.

#### *Clinical presentation of BA patients following clinical features collected from the patients charts.*

Serum total bilirubin TB (mg/dl), aspartate amino-transferase AST (IU/L), alanine aminotransferase ALT (IU/L), and gamma glutamyl transpeptidase GGTP (IU/L) were measured in the Osaka University Hospital laboratory and analyzed.

#### *Classification of esophageal and liver fibrosis*

Esophagogastroduodenoscopy (EGD) and liver biopsy was performed at same time in Post-KP and Post-LT patients, as a routine examination to evaluate the progression of esophageal varices and liver fibrosis, respectively. The grade of esophageal varix was classified according to Japan Society for Portal Hypertension classification, as follows: F0, No varicose appearance; F1, Straight, small-caliber varices; F2: Moderately enlarged, beady

varices; F3: Markedly enlarged, nodular or tumor-shaped varices [12], and the grade of liver fibrosis was classified according to new Inuyama classification, as follow: F0, no fibrosis; F1, fibrous portal expansion; F2, bridging fibrosis; F3, bridging fibrosis with architectural distortion; and F4, liver cirrhosis [13].

### *Statistical analysis*

Data were entered into the SPSS (Chicago IL) 16.0 software package. Mann-Whitney U test, Kruskal-Wallis test, Wilcoxon signed rank test, and Spearman's correlation coefficient were used. Significance levels were set at  $p$  less than .05.

### **Results**

To investigate the alteration of apelin mRNA expression during the progression of BA, we analyzed the mRNA levels in the livers of BA patients at different stages.

The results showed that apelin mRNA expression was significantly higher in LT group than control, KP, Post-KP and Post-LT groups (Fig 1) (LT versus CO,  $p < 0.05$ ; LT versus KP,  $p < 0.01$ ; LT versus Post-KP,  $p < 0.001$ , and LT versus Post-LT,  $p < 0.001$ ).

The alteration of apelin expression during the progression of BA in the same patients (see the details in Table 1) was also analyzed. The result showed that apelin mRNA expression levels in the samples from the patients who underwent LT (patient 1, 3, 6 and 7, Fig 2) were nearly 10-fold increased compared with in the sample taken from KP and Post-KP but no statistical significance ( $p = 0.068$ ).

In order to assess apelin mRNA expression during liver fibrosis progression, 42 liver samples from BA patients of KP, Post-KP and LT were subjected to real-time PCR. Apelin mRNA expression was significantly higher at fibrosis stage 4 than at stage 0-1 and stage 2-3 according to New Inuyama, (stage 4 versus stage0-1,  $p < 0.05$ ; stage 4 versus stage2-3,  $p < 0.01$ ) (Fig 3).

Endoscopy was performed in 32 patients to evaluate the grade of esophageal varices. Apelin mRNA expression was significantly correlated with grade of esophageal varices, a grade-dependent upregulation of apelin mRNA expression increased significantly during the progression of esophageal varices (grade2-3 (F2-3) versus grade0 (F0),  $p < 0.01$ ; grade1 (F1)

versus grade0 (F0) and grade2-3 (F2-3),  $p < 0.05$ ).

Moreover, when apelin mRNA levels in liver tissue of those patients were compared with their esophageal grade, a significant correlation was found ( $r_s = 0.522$ ,  $p < 0.01$ ) (Fig 5).

Of the routine liver function tests, relative apelin RNA expression level significantly correlate with serum TB level ( $r_s = 0.520$   $P < 0.001$ , Table 2), but did not correlate with the value of AST, ALT or GGP (Table 2)

In an attempt to identify the cellular source of the altered apelin expression, we performed histological immunolocalization of apelin and APJ in liver tissue in normal control and all the stages of BA.

In control liver tissue, apelin was mainly localized in the perivenular areas (large portal vein and ventral vein), and slightly detected in the sinusoid and parenchyma, whereas APJ was almost undetected in perivenular areas but mainly in the parenchyma. In KP and Post-KP liver tissue, apelin was observed mainly in perivenular areas and capillaries and moderately in the sinusoids, whereas a marked decrease APJ expression was detectable in Post-KP stage. In LT liver tissue, intense apelin immunoreactivity was detected mainly in perivenular, sinusoidal and parenchyma areas (details summarized in Table 2).

## Discussion

Apelin and its receptor system has attracted widespread research interest and its pathophysiological roles are gradually emerging [14, 15]. Recent studies showed apelin plays an important role in liver disease [16, 17]. Here we show for the first time that apelin mRNA expresses in liver tissue of BA patients. The results showed that the apelin mRNA expression was significant higher in end-stage (LT) than controls, early-stage (KP, Post-KP) of BA, and decreased to normal compared with control after liver transplantation. These findings suggest that apelin may be a significant factor for prediction poor prognosis (need liver transplantation) of BA. Although statistic analysis showed no significant alternation on apelin mRNA expression in the same patients during the progression of BA that may be due to the limited cases, expression from those 4 patients were much higher in their end stage than their early stage. In

addition, our study showed that apelin mRNA expression had not significant correlation with liver function (i.e. AST, ALT, and GGP) except TB. This finding indicates that maybe TB plays more important role in predicting poor prognosis of BA than any other indicator of liver function. The development of the KP improved the prognosis for children with biliary atresia [18-21]. Despite the increasing number of patients who survive jaundice free for an indefinite period after KP, liver fibrosis progresses in many patients [22] [23] and risk of gastrointestinal (GI) bleeding due to HP make BA continue to remain the leading indication for pediatric liver transplantation.

Fibrosis is a response of the injured liver where tissular healing results from the activation of several cell types and in which numerous cell mediators are involved [24]. In agreement with previous studies [25] demonstrating that patients with cirrhosis showed a significant increased apelin mRNA expression level, the current study has demonstrated that it is overexpressed in cirrhotic liver of BA patients.

Portal hypertension is a serious complication of chronic liver diseases and a leading cause of liver transplantation and mortality worldwide [26]. One of the characteristics of PH is the formation of an extensive network of portosystemic collateral vessels that include the gastroesophageal varices, which are the most important consequences of chronic liver disease [26]. Virtually, all children with BA are at risk for portal hypertension secondary to cirrhosis, regardless of the result of KP. Uncontrollable gastrointestinal bleeding due to the portal hypertension is life threatening [27-30] and regarded as the indication of LT [31, 32]

Apelin has been reported to play a role in vascular remodeling and in increased portal hypertension in cirrhosis [10]. Our study showed a grade-dependent upregulation of apelin mRNA expression during the progression of esophageal varices in BA. This result indicates that apelin plays an important role in increased portal hypertension in BA.

All the results above about apelin mRNA expression parallel the immunohistochemistry findings that apelin expression was marked in the proliferated arterial capillaries in the peripheral region of fibrous septa and in the sinusoids in the regenerative nodule surrounded by broad fibrous septa in the liver tissue from BA patients with cirrhosis and high grade of esophageal varices

In conclusion, our study suggests: 1) high level of apelin expression is a characteristic finding



in end-stage of BA, and 2) apelin expression level accurately reflects the severity of hepatic fibrosis and esophageal varices and therefore could be used as a prognostic factor to estimate the timing of liver transplantation in BA patients

Fig 1 Relative mRNA expression in livers of BA patient.

\*p < 0.05, \*\*p < 0.01, \*\*\* p < 0.001

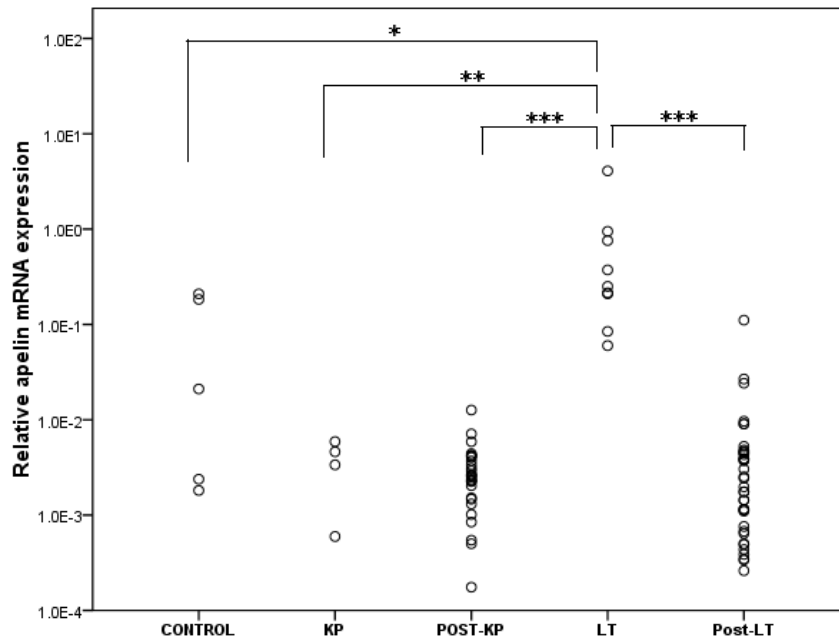


Fig 2 Alteration of apelin mRNA expression in the same patients during the progression of BA.

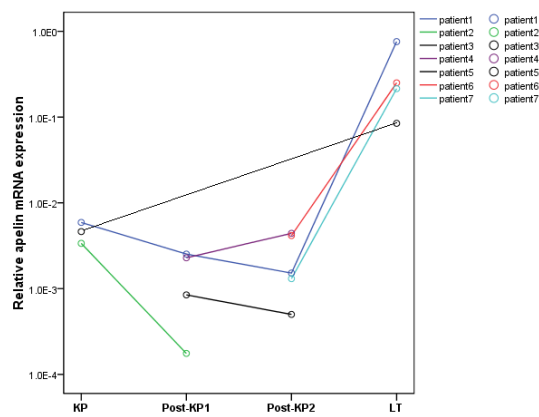


Fig 3 Apelin increases with fibrosis progression in BA patients

Number of patients per group is shown above each bar. Boxes encompass the 25th to 75th percentile, horizontal lines represent the median (50th percentile), and whiskers extend to the smallest and highest values. \*  $p < 0.05$ , \*\*  $p < 0.01$ .

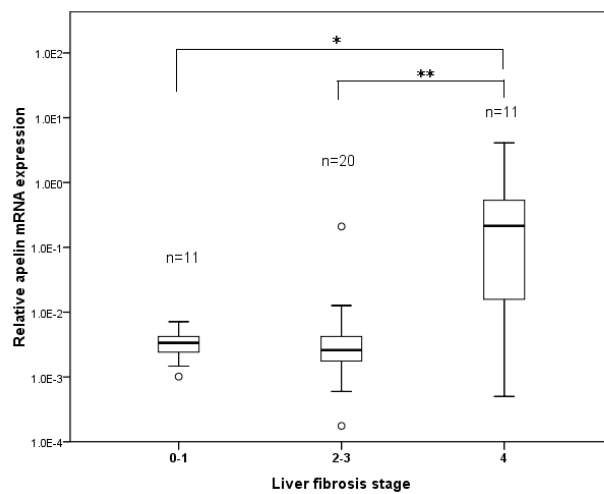
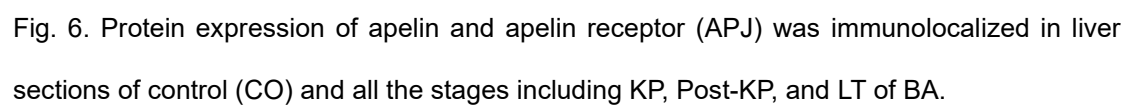
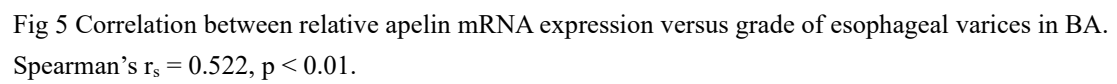


Fig 4 Apelin mRNA expression with esophageal varices progression in BA patients.

\*  $p < 0.05$ , \*\*  $p < 0.01$ .



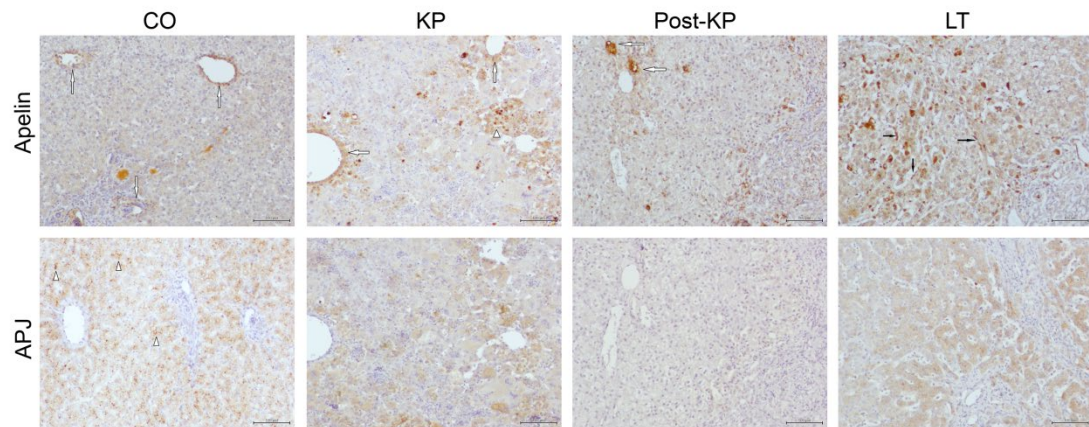


Table 1 Details of BA patients who took more than one operation

Patients	Sex	Age when the operation was performed		
		KP	Post-KP	LT
1	M	67 days	191/331 days*	518 days
2	F	74 days	383 days	—
3	M	88 days	—	242 days
4	F	—**	4.2/4.6 years*	—
5	F	—**	3.1/4.1 years*	—
6	F	—**	183 days	265 days
7	F	—**	408 days	977 days

\* Patient took needle liver biopsy after KP for twice

\*\* Patient took KP in other institution

Table 3 Intensity of apelin and APJ immunostaining in different stage of BA

	CO		KP		Post-KP		LT	
	Apelin	APJ	Apelin	APJ	Apelin	APJ	Apelin	APJ
Perivenular area	+	—	+	—	+	—	+	—
capillary	±	—	+	—	+	—	+++	—
Sinusoidal area	±	±	+	—	+	—	+++	±
parenchyma	+	++	+	+	+	±	++	+

Table 2 Correlation coefficient in BA patients\*

	Correlation coefficient	Statistical significance
TB	0.527	< 0.0001
AST	0.095	n.s.
ALT	0.109	n.s.
GGP	-0.238	n.s.

Spearman correlation test.

## References

- [1] Tatemoto K, Hosoya M, Habata Y, et al. Isolation and characterization of a novel endogenous peptide ligand for the human APJ receptor. Biochem Biophys Res Commun 1998; 251:471-476
- [2] O'Dowd BF, Heiber M, Chan A, et al. A human gene that shows identity with the gene

- encoding the angiotensin receptor is located on chromosome 11. *Gene* 1993;136(1-2):355-60
- [3] Ishida J, Hashimoto T, Hashimoto Y, et al. Regulatory roles for APJ, a seven-transmembrane receptor related to angiotensin-type 1 receptor in blood pressure in vivo. *J Biol Chem* 2004; 279:26274-26279
- [4] Szokodi I, Tavi P, Foldes G, et al. Apelin, the novel endogenous ligand of the orphan receptor APJ, regulates cardiac contractility. *Circ Res* 2002; 91:434-440
- [5] Boucher J, Masri B, Daviaud D, et al. Apelin, a newly identified adipokine up-regulated by insulin and obesity. *Endocrinology* 2005; 146: 1764–1771
- [6] Wang G, Anini Y, Wei W, et al. Apelin, a new enteric peptide: localization in the gastrointestinal tract, ontogeny, and stimulation of gastric cell proliferation and of cholecystokinin secretion. *Endocrinology* 2004; 145: 1342–1348.
- [7] De Mota N, Reaux-Le Goazigo A, El Messari S, et al. Apelin, a potent diuretic neuropeptide counteracting vasopressin actions through inhibition of vasopressin neuron activity and vasopressin release. *Proceedings of the National Academy of Sciences* 2004; 101: 10464–10469
- [8] Hiroaki Y, Masaya O, Kazunori Y, et al. Overexpression of apelin receptor (APJ/AGTRL1) on hepatic stellate cells and sinusoidal angiogenesis in human cirrhotic liver. *J Gastroenterol* 2011; 46:222-231
- [9] Principe A, Melgar-Lesmes P, Fernández-Varo G, et al. The hepatic apelin system: a new therapeutic target for liver disease. *Hepatology* 2008; 48:1193–1201
- [10] Yokomori H, Oda M, Yoshimura K, et al. Enhanced expressions of apelin on proliferative hepatic arterial capillaries in human cirrhotic liver. *Hepatol Res.* 2012;42(5):508-14
- [11] Okazaki T, Kobayashi H, Yamataka A, et al. Long-term postsurgical outcome of biliary atresia. *J Pediatr Surg* 1999; 34:312-5
- [12] Tajiri T, Yoshida H, Obara K, et al. General rules for recording endoscopic findings of esophagogastric varices (2nd edition). *Dig Endosc* 2010; 22: 1—9.
- [13] Ichida F, Tsuji T, Omata M, et al. New Inuyama classification for histological assessment of chronic hepatitis. *Hepatol Commun* 1996; 6:112-9.
- [14] Lee DK, Cheng R, Nguyen T, et al. Characterization of apelin, the ligand for the APJ receptor. *J Neurochem* 2000;74:34-41
- [15] Hosoya M, Kawamata Y, Fukusumi S, et al. Molecular and functional characteristics of APJ. Tissue distribution of mRNA and interaction with the endogenous ligand apelin. *J Biol Chem* 2000;275:21061-21067.
- [16] Alessandro P, Pedro M, Guillermo FV, et al. The hepatic apelin system: a new therapeutic target for liver disease. *Hepatology* 2008; 48:1193-1201
- [17] Hiroaki Y, Masaya O, Kazunori Y, et al. Enhanced expression of apelin on proliferative hepatic arterial capillaries in human cirrhotic liver. *Hepatology research* 2012;42: 508-514
- [18] Kasai M. Treatment of biliary atresia with special reference to hepatic portoenterostomy and its modifications. *Prog Pediatr Surg* 1974;6:5-52.
- [19] Karrer FM, Price MR, Bensard DD, Sokol RJ, Narkewicz MR, Smith DJ, et al. Long-term results with the Kasai operation for biliary atresia. *Arch Surg* 1996;131:493-6.
- [20] Ohi R, Nio M, Chiba T, Endo N, Goto M, Ibrahim M. Long-term follow-up after surgery for patients with biliary atresia. *J Pediatr Surg* 1990;25:442-5.

- [21] Tagge DU, Tagge EP, Drongowski RA, Oldham KT, Coran AG. A longterm experience with biliary atresia. *Ann Surg* 1991;214:590-8.
- [22] Gautier M, Valayer J, Odièvre M, et al. Histological liver evaluation 5 years after surgery for extrahepatic biliary atresia: a study of 20 cases. *J Pediatr Surg* 1984;19:263–268
- [23] Lykavieris P, Chardot C, Sokhn M, et al. Outcome in adulthood of biliary atresia: a study of 63 patients who survived for over 20 years with their native liver. *Hepatology* 2005;41:366–371.
- [24] Friedman SL. Mechanisms of hepatic fibrogenesis. *Gastroenterology* 2008.134:1655-1669.
- [25] Pedro M-L, Gregori C, Montserrat P, et al. Apelin mediates the induction of profibrogenic genes in human hepatic stellate cells. *Endocrinology* 2010; 151(11): 5306-14
- [26] Bosch J, Pizcueta P, Feu F, et al. Pathophysiology of portal hypertension. *Gastroenterol Clin North Am* 1992; 21:1-13
- [27]. Ernest van Heurn LW, Saing H, Tam PKH. Portoenterostomy for biliary atresia: long-term survival and prognosis after esophageal variceal bleeding. *J Pediatr Surg* 2004;39:6–9.
- [28] Park A, Cwikiel W. Emergent treatment of variceal bleeding in two infants. *Acta Radiol* 2008;49:951–954.
- [29] Tagge DU, Tagge EP, Drongowski RA, et al. A long-term experience with biliary atresia. Reassessment of prognostic factors. *Ann Surg* 1991;214:590–598.
- [30] Ohkohchi N, Chiba T, Ohi R, et al. Long-term follow-up study of patients with cholangitis after successful Kasai operation in biliary atresia; selection of recipients for liver transplantation. *J Pediatr Gastroenterol Nutr* 1989;9:416–420.
- [31] Shinkai M, Ohhama Y, Take H et al: Evaluation of the PELD risk score as a severity index of biliary atresia. *J Pediatr Surg* 2003; 38: 1001–4
- [32] de Vries W, de Langen ZJ, Aronson DC et al: Mortality of biliary atresia in children not undergoing liver transplantation in the Netherlands. *Pediatr Transplant*, 2011; 15: 176–83