Int J Clin Exp Pathol 2016;9(5):5077-5086
www.ijcep.com /ISSN:1936-2625/IJCEP0018058

Original Article

SOX9-positive hepatocytes mediate the progression of ductular reaction in biliary atresia

Daiki Yoshii[1], Yuji Yokouchi[2], Hiroko Suda[3], Yukihiro Inomata[4]

[1]Department of Pediatric Surgery and Transplantation, Graduate School of Medical Sciences, Kumamoto University, Kumamoto, Japan; [2]Department of Stem Cell Research, Fukushima Medical University, Fukushima, Japan; [3]Department of Pediatric Surgery and Transplantation, Kumamoto University Hospital, Kumamoto, Japan; [4]Department of Pediatric Surgery and Transplantation, Faculty of Life Sciences, Kumamoto University, Kumamoto, Japan

Received October 18, 2015; Accepted March 22, 2016; Epub May 1, 2016; Published May 15, 2016

Abstract: Ductular reaction (DR) is seen in liver diseases as an abnormal proliferation of cells with a biliary phenotype; however, the mechanism underlying this condition is controversial. We have previously demonstrated ectopic expression of SOX9, a marker of biliary cells, on HepPar1-positive hepatocytes in biliary atresia (BA). Notch signaling has recently been reported to be involved in the biliary conversion of hepatocytes *in vivo*. The aims of this study, using BA livers, were to elucidate the involvement of ectopic SOX9 expression with DR and the association between Notch signaling and DR progression. Formalin-fixed paraffin embedded liver sections from 47 BA livers were used. Liver specimens from three living liver transplant donors were included as control. Two Alagille syndrome (AGS) livers were used for investigating the involvement of Notch signaling. The medical records of the patients were retrospectively reviewed. Image and data processing were performed using ImageJ software. Progressive cholestatic liver injury after Kasai portoenterostomy led to decreasing numbers of SOX9+HepPar1+ cells and an increasing CK19+ area. SOX9+HepPar1+ cell numbers were significantly negatively correlated with CK19+ area size (r=-0.6, P=0.002) at the time of liver transplant. Immunohistochemical analysis using AGS liver, which has defective Notch signaling, showed remarkable accumulation of SOX9+HepPar1+ cells and a reduction in the number of CK19+ cells. The progression of DR in BA liver, characterized by increasing CK19+ area, is mediated by SOX9+HepPar1+ cells, and Notch signaling may be required for the progression of DR.

Keywords: SOX9, ductular reaction, biliary atresia, notch signaling, Alagille syndrome

Introduction

The liver has a remarkable capacity for hepatocyte self-renewal to enable homeostasis to be maintained [1]. However, impaired self-renewal capacity following liver injury leads to abnormal proliferation of cells with a biliary phenotypic, such as cytokeratin19-positive (CK19+) reactive ductular cells (RDCs). This pathological condition is known as ductular reaction (DR) [2, 3]. It is thought that DR aims to repair and compensate for anatomical and functional loss caused by liver injury [4]; however, DR is also thought to have a crucial role in the etiology of fibrosis [5]. Three possible candidates for the origin of DR cells have been reported; preexisting cholangiocytes, hepatic stem/progenitor cells and hepatocytes [3, 6, 7], but the exact origin is still unknown. Therefore, understanding the mechanism of DR is essential for controlling the pathological conditions of liver disease. Sex-determining region Y-box9 (*SOX9*) was identified as the gene responsible for the human haploinsufficiency disease, campomelic dysplasia (OMIM #114290) [8, 9]. In liver, SOX9 is the earliest biliary marker and regulates the maturation of bile ducts during embryonic development [10]. Also, the SOX9+ liver progenitor zone continuously supplies hepatocytes during liver regeneration [11]. Thus, SOX9 has an important function in bile duct generation and liver regeneration. Biliary atresia (BA) is a rare infantile disease characterized by bile duct obliteration with unknown pathogenesis [12]. Kasai portoenterostomy (KP) is usually performed in the early infantile period, but subse-

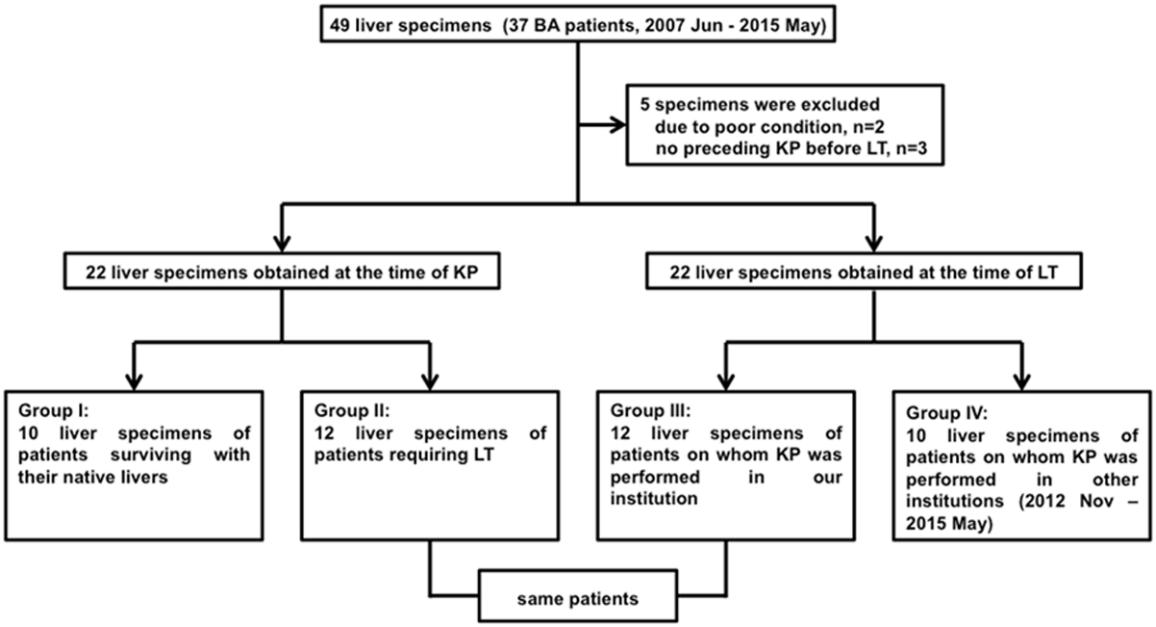


Figure 1. Breakdown for the liver specimens. A total of 49 liver specimens from 37 BA patients were enrolled. Five specimens were excluded from this study. The liver specimens from 12 patients were analyzed both at the time of KP and LT (groups II and III).

quent liver transplantation (LT) is necessary in cases with failed KP [12]. DR is a characteristic pathological feature of BA [13]. We previously reported ectopic SOX9 expression on HepPar1+ hepatocytes in BA. Furthermore, chronological change in the expression of SOX9 and CK19 from the time of KP to LT suggested that SOX9+HepPar1+ cells have the possibility of emerging earlier than CK19+ RDCs [14]. Thus, we hypothesized that SOX9+HepPar1+ cells are involved in the emergence of CK19+ RDCs. The aim of this study was to elucidate the association of SOX9+HepPar1+ cells with the progression of DR in BA liver, and to shed light on the mechanism of DR progression.

Here, we attempted to confirm the relationship between SOX9+HepPar1+ cells and DR. We then attempted to confirm whether the Notch signaling, a key regulator in normal biliary development [15], is involved in the progression of DR using Alagille syndrome (AGS) liver. We focused on Notch signaling because activation of Notch signaling mediates the conversion of hepatocytes to a biliary phenotype *in vivo* [16, 17]. Also SOX9 is a direct target of Notch [16]. AGS is a dominantly inherited disorder characterized by interlobular bile duct paucity as a consequence of mutations in the genes encoding *JAG1* [18], a Notch receptor ligand , or *NOTCH2*, a Notch receptor [19]. Our results indicate that ectopic SOX9 expression has a close relationship with DR progression, and suggest that Notch signaling may be required for the progression of DR during cholestatic liver injury.

Materials and methods

Forty nine liver specimens from 37 BA patients who underwent KP and/or LT in our institution between January 2007 and May 2015 were recruited (**Figure 1**). Each specimen was divided into four groups. Group I, 10 liver specimens obtained at the time of KP from patients living with a native liver; group II, 12 specimens obtained at KP from patients requiring LT; group III, 12 specimens obtained at LT from patients who had undergone KP in our institution; group IV, 10 specimens obtained at LT from patients who had undergone KP in other institutions. Liver specimens of groups II and III were obtained from the same patients. All BA patients were type 3 (atresia at the porta hepatis, the most common type) [12]. Three living donors for LT and two AGS patients were included for pathological comparison with BA patients. The AGS patients were confirmed by identification of *JAG1* mutations; one was c.2408 G>A (p.W803X), the other was c.222

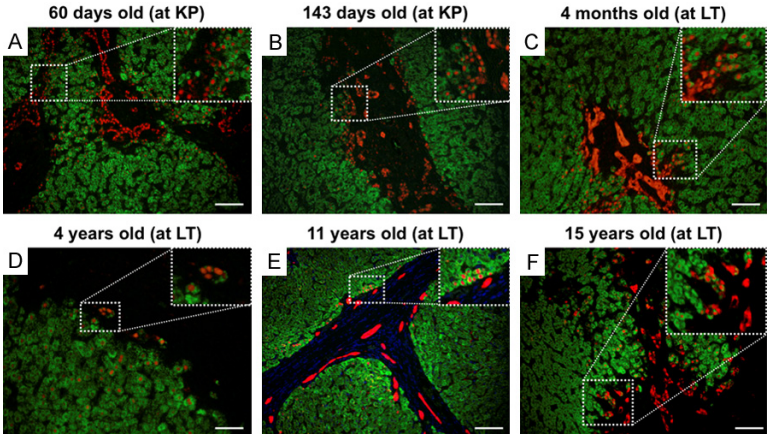


Figure 2. Duct-like structures consist of SOX9+HepPar1+ cells in BA patients of various ages. The duct-like structures are seen in periportal fields or at the periphery of hepatic lobules. Most duct structures consisted of SOX9+HepPar- cells. Framed squares show the duct-like structures under twofold magnification. SOX9: *red*. HepPar1: *green*. Scale bars: 100 μm (A-C, E and F); 50 μm (D).

C>A (p.Y74X). Two BA patients were excluded because of a poor state of specimen preservation, and three patients who underwent LT without preceding KP were also excluded. Specimens were collected by wedge resection from the periphery of native livers. Medical records were retrospectively reviewed. Collected clinical data were age, and levels of serum total bilirubin (T-Bil), aspartate transaminase (AST), alanine transaminase (ALT), gamma-glutamyl transferase (γ-GTP), total protein (TP), choline esterase (ChE), white blood cells (WBC), platelets (Plt), international normalized ratio of prothrombin time (PT-INR) and fibrosis (F) staging. F staging was evaluated by New Inuyama classification as follows: F0, no portal fibrosis; F1, fibrosis portal expansion; F2, bridging fibrosis; F3, bridging fibrosis with lobular distortion; and F4, cirrhosis [20]. This study was approved by the institutional review boards at Kumamoto University Hospital, and written informed consent was obtained from each patient.

Immunofluorescence staining (IF)

Specimens were fixed at room temperature for at least 72 hours in 10% formalin (134-10047; Wako Pure Chemical Industries, Ltd., Osaka, Japan) and embedded in paraffin (7810; Sakura Finetek Japan Co., Ltd., Tokyo, Japan). Specimens were then sectioned at 5 μm. The IF protocol used was as described previously [14]. The primary antibodies and concentrations used were: rabbit anti-SOX9 at 1:200 (AB5535; Merck Millipore, Billerica, MA, USA), mouse anti-CK19 at 1:100 (NCL-CK19; Leica Biosystems, Wetzlar, Germany) and mouse anti-HepPar1 at 1:35 (M7158; Dako Japan Co., Ltd., Tokyo, Japan). Nuclei were counterstained with 4',6-diamidine-2'-phenylindole dihydrochloride (DAPI) (SCR-38448; dianova GmbH, Hambrug, Germany).

Image processing

Immunostained sections were photographed with an Olympus BX51 microscope at 20× magnification. To quantify the immunostaining, at least six non-overlapping randomly selected portal areas were taken for each patient. Immunostaining images were processed by Paintshop® Pro 12 (COREL™). The following processes were performed using ImageJ 1.46 software (National Institutes of Health, Bethesda, MD, USA). Images of DAPI and SOX9 were converted to 8-bit grayscale to adjust the threshold. Threshold values were given by auto thresholding. Watershed segmentation was then performed, which is a method for automatically separating particles to avoid underestimation due to aggregation of cells. The numbers of SOX9+ and DAPI+ cells were counted automatically. The size of the lower limit of counted cells was set at 20 pixel^2. SOX9+ HepPar1+ and SOX9+HepPar-negative (SOX9+ HepPar-) cells were visually counted. The SOX9+HepPar1+ ratio is the percentage of the SOX9+HepPar1+ cell number to the DAPI+ cell number in a HepPar1+ field. The SOX9+HepPar1- ratio is the percentage of the SOX9+HepPar1- cell number to the DAPI+ cell number in a field. Images of CK19 staining were split into three 8-bit grayscale images by "Split Channels" and the threshold of the selected image from the three was adjusted. The applied threshold value was 30 added to a value given by auto thresholding. This was because compensated images were more reflective of original images. The CK19+ ratio is the percentage of positive pixels to total pixels in a field.

Table 1. Clinical data of BA patients at the time of KP and LT

	Group II (at KP, n=12)	Group III (at LT, n=12)	*P* value
Age at KP (days)	70.5 (29-143)	Same as left	NA
Age at LT (year)	NA	0.5 (0.3-0.7)	NA
T-Bil (mg/dL)	9.2 (4.8-10.3)	12.0 (3.1-42.7)	0.16†
ALT (U/L)	89.5 (26-272)	132 (30-714)	0.27†
γ-GTP (U/L)	367 (110-1025)	180 (24-513)	0.06†
TP (g/dL)	5.8 (5.1-6.9)	5.9 (4.2-7.4)	0.88†
ChE (U/L)	292 (201-397)	115 (61-212)	0.002†
WBC (/μL)	12600 (8100-19200)	11500 (5400-18400)	0.33†
Plt ($\times 10^3$/μL)	340 (171-721)	167 (115-354)	0.004†
PT-INR	1.0 (0.9-1.4)	1.4 (1.1-1.8)	0.003†
F staging	3 (1-4)	4 (3-4)	0.007‡

NA, not applicable. Data are shown as medians (ranges). †Wilcoxon signed-rank test, ‡Fisher's exact test.

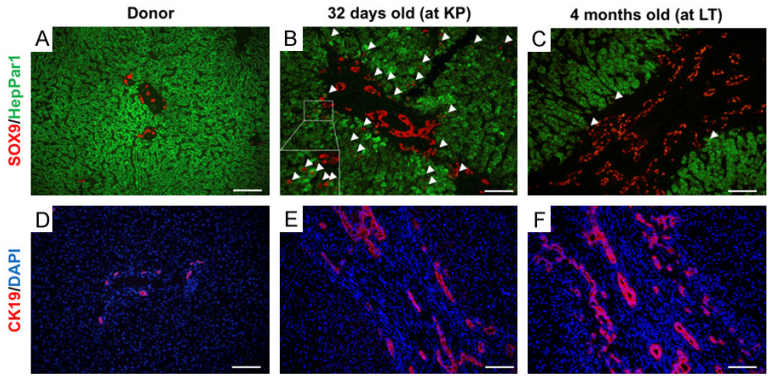


Figure 3. Immunofluorescence staining of a living donor and BA livers. Many more SOX9+HepPar1+ cells are found at KP than at LT, and are rarely seen in a donor liver. Arrowheads show SOX9+HepPar1+ cells. More SOX9+HepPar1- cells and CK19+ RDCs are observed at LT than at KP. Scale bars: 100 μm.

Statistical analysis

All data in tables are shown as median values and ranges. The Mann-Whitney U-test and the Wilcoxon signed-rank test were used to test comparisons between groups. Categorical variables were compared with Fisher's exact test. The relationship between valuables was assessed using Pearson's correlation coefficient or Spearman's rank correlation coefficient. A receiver operating characteristic (ROC) curve was obtained for determination of the cut-off value. The value was determined by the closest point on the ROC curve to the upper left corner. Native liver survival rate was estimated using the Kaplan-Meier method and the log rank test. All tests were two sided, and $P \leq 0.05$ was considered statistically significant. Statistical analyses were performed using PASW Statistics 18.0 software (SPSS, Inc., Chicago, IL, USA).

Results

Duct-like structures consist of SOX9+HepPar1+ cells

To investigate a potential pathological role of SOX9+HepPar1+ cells, we performed double IF for SOX9 and HepPar1 in BA liver obtained at the time of KP and LT. SOX9 is a biliary marker [10] and is also expressed in RDCs [14]. Most duct structures were composed of SOX9+HepPar1- cells (**Figure 2**). However, in periportal fields or at the periphery of hepatic lobules, duct-like structures consisting of SOX9+HepPar1+ cells were found (**Figure 2**). This finding was recognized at various ages (**Figure 2**), but never in living donors. This finding suggests that SOX9+HepPar1+ cells could be associated with the emergence of duct structures in BA.

Ectopic SOX9 expression may expand prior to the progression of DR and decrease under progressive cholestatic liver injury after KP

To confirm the emergence of ectopic SOX9 expression preceding the proliferation of CK19+ cells in BA livers, we performed IF and a chronological comparison using group II (at KP, n=12) and group III (at LT, n=12). The two groups were derived from the same patients. Prior to histological analysis, we retrospectively checked clinical data (**Table 1**). All patients who required LT and who had undergone KP in our institution were less than 1 year old during the study period. There were no improvements after KP in liver function at the time of LT as indicated by T-Bil, ALT and γ-GTP. Furthermore, ChE, PT-INR and F staging were significantly worsened at LT (P=0.002, P=0.003 and P=0.007, respectively). These data indicate that BA patients in group III had progressive cholestatic liver injury after KP.

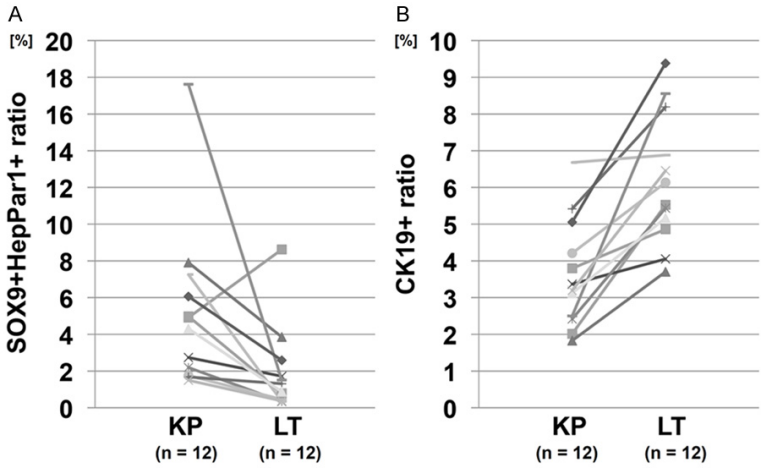


Figure 4. Transition of SOX9+HepPar1+ ratios (A) and CK19+ ratios (B) from the time at KP to LT. Almost all the SOX9+HepPar1+ ratios were decreased at the time of LT, whereas all of the CK19+ ratios were increased at the time of LT.

Representative IF images of the BA patient are shown with those of a living donor (**Figure 3**). Many more SOX9+HepPar1+ cells were observed at KP than at LT. In contrast, more CK19+ and SOX9+HepPar1- cells were observed at LT than at KP. SOX9+HepPar1+ cells were rarely seen in donors (**Figure 3**). The Wilcoxon signed-rank test (group II vs III) showed that the SOX9+HepPar1+ ratio at the time of LT was significantly decreased compared with that at the time of KP (P=0.02), whereas CK19+ and SOX9+HepPar1- ratios at the time of LT were significantly increased compared with those at the time of KP (P=0.002 and P=0.03, respectively) (**Table 2**). In addition, all SOX9+HepPar1+ ratios decreased except in one patient, whereas all CK19+ ratios increased (**Figure 4**). These data indicate that progressive cholestatic liver injury facilitates not only DR progression, as shown by the increased number of CK19+ and SOX9+HepPar1- cells but also by the decreased number of SOX9+ HepPar1+ cells. In addition, these data suggest that the emergence of SOX9+HepPer1+ cells may precede the proliferation of CK19+ cells in BA liver.

Ectopic expression of SOX9 has a close relationship with DR progression at LT

To confirm the relationship between the level of clinical cholestasis after KP and the populations of SOX9+HepPar1+, SOX9+HepPar1- and CK19+ cells, we analyzed the statistical correlations between T-Bil and the ratios using all liver specimens at the time of LT (groups III and IV). Although the SOX9+ HepPar1+ ratio had no significant correlation with T-Bil levels (r=-0.3, P=0.23, data not shown), SOX9+HepPar1- and CK19+ ratios positively correlated with T-Bil levels (r=0.7, P=0.001; r=0.5, P=0.009, respectively) (**Figure 5A** and **5B**). The positive correlations between SOX9+HepPar1- and CK19+ ratios and T-Bil levels indicate that proliferation of cells with a biliary phenotype are involved in the severity of cholestasis.

Next, to investigate whether ectopic SOX9 expression is involved in DR progression, we determined the pathological relationship between ectopic SOX9 expression and the proliferation of biliary phenotypic cells at LT. We assessed the statistical correlations between the SOX9+HepPar1+ ratio and SOX9+HepPar1- and CK19+ ratios. The SOX9+HepPar1+ ratio had a significantly negative correlation with the SOX9+HepPar1- ratio (r=-0.5, P=0.03) and the CK19+ ratio (r=-0.6, P=0.002) (**Figure 5C** and **5D**). The negative correlations between the SOX9+HepPar1+ ratio and both SOX9+HepPar1- and CK19+ ratios indicate that the SOX9+ HepPar1+ ratio may be associated with DR progression.

Notch signaling may be required for the SOX9+HepPar1+ cell-mediated progression of DR

To elucidate whether Notch signaling is involved in DR progression, we performed IF with AGS liver sections at the time of LT. The numbers of CK19+ cells and interlobular bile ducts that consisted of CK19+ cells were small in spite of sustained severe cholestasis (**Figure 6A**, **6B** and **6E**). Surprisingly, drastic expansions of SOX9+HepPar1+ cells were confirmed in liver lobes (**Figure 6C** and **6D**). These findings indicate that defective Notch signaling can induce the accumulation of SOX9+HepPar1+ cells and reduce the number of CK19+ cells despite

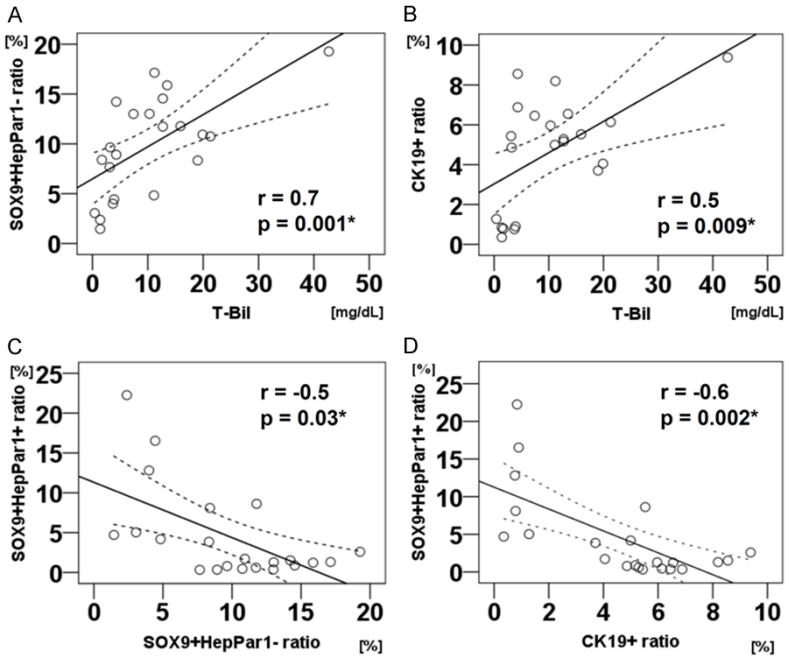
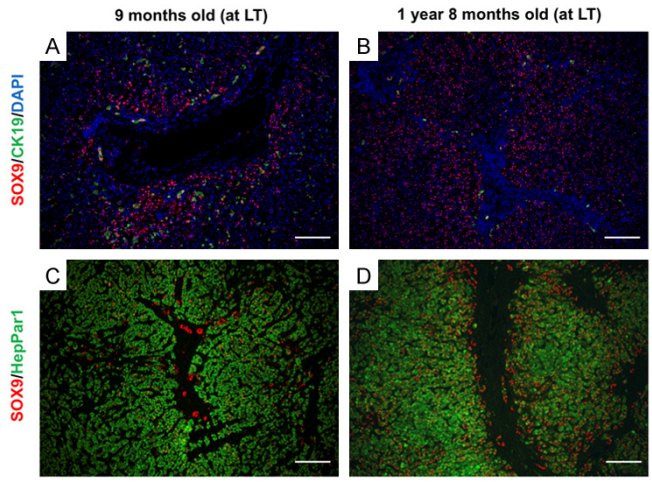


Figure 5. Correlations between Parameters. A, B. Correlation with the level of T-Bil. C, D. Correlation between SOX9+HepPar1+ ratio and SOX9+HepPar1- and CK19+ ratios. Dotted lines indicate 95% confidence interval. *Spearman's rank correlation coefficient.



E

	SOX9+HepPar1+ ratio (%)	CK19+ ratio (%)	T-Bil (mg/dL)	ALT (U/L)	T-BA (μmol/L)	γ-GTP (U/L)
AGS (9 m old)	38.6	1.2	18.9	36	280	234
AGS (1y8m old))	49.9	0.4	12	126	352	252

Figure 6. Immunofluorescence staining and clinical data of AGS patients at the time of LT. A small number of CK19+ cells is confirmed despite sever cholestasis (A, B and E), whereas a quite large number of SOX9+HepPar1+ cells is confirmed in AGS livers (C, D). Scale bars: 200 μm. m, months; y, year.

severe cholestasis. This suggests that Notch signaling may be required for SOX9+HepPar1+ cells to be involved in DR progression.

Discussion

The findings of this study using BA liver indicated that SOX9+HepPar1+ cells can have a role in mediating DR progression following cholestatic liver injury. Moreover, immunohistochemical findings using AGS liver suggested that Notch signaling may be needed in SOX9+HepPar1+ cells for the progression of DR. From this study, the importance of SOX9+HepPar1+ cells is recognized.

The most likely candidate for the origin of SOX9+HepPar1+ cells is hepatocytes. This is because the chronological changes of a decreasing SOX9+HepPar1+ ratio and increasing SOX9+HepPar1- and CK19+ ratios under progressive cholestatic liver injury after KP (**Table 2**) suggest that cells with a biliary phenotype are derived from cells with a hepatocytic phenotype. This speculation is consistent with recent *in vivo* reports demonstrating that intermediate cells, which have both hepatocytic and biliary phenotypes, originate from hepatocytes [17, 21]. Moreover, Sox9 is an early marker of hepatocyte-to-biliary cell conversion in mouse [17, 22]. In this study, we cannot exclude the possibility that a subpopulation of SOX9+HepPar1+ cells originate from hepatic progenitor cells (HPCs). However, HPCs do not effectively produce mature hepatocytes in mouse injury models [23, 24]. Moreover, AGS livers have many SOX9+HepPer1+ cells (**Figure 6C-E**) in spite of a near absence of HPCs and RDCs [13]. Thus,

Table 2. Immunohistochemical analysis data of BA patients at the time of KP and LT

	Donor (n=3)	Group II (at KP, n=12)	Group III (at LT, n=12)	*P* value
SOX9+HepPar1+ ratio (%)	0.0 (0.0-0.5)	4.6 (1.5-17.6)	1.1 (0.3-8.6)	0.02
SOX9+HepPar1- ratio (%)	1.6 (1.3-2.6)	6.9 (4.0-15.3)	11.3 (7.7-19.3)	0.03
CK19+ ratio (%)	0.5 (0.5-0.7)	3.3 (1.8-6.7)	5.8 (3.7-9.4)	0.002

Data are shown as medians (ranges). Donor data is shown as control. Wilcoxon signed-rank test is used to compare between groups II and III.

Table 3. Comparative data between native liver survivors (group I) and non-native liver survivors (group II)

	Group I (at KP, n=10)	Group II (at LT, n=12)	*P* value
Age at of KP (days)	66 (38-101)	70.5 (29-143)	0.87†
T-Bil (mg/dL)	9.1 (7.9-11.6)	9.5 (4.8-10.3)	1.0†
ALT (U/L)	105 (21-264)	89.5 (26-272)	1.0†
γ-GTP (U/L)	607 (139-1896)	367 (110-1025)	0.26†
TP (g/dL)	5.6 (4.9-6.4)	5.8 (5.1-6.9)	0.67†
ChE (U/L)	265 (141-359)	292 (201-397)	0.15†
WBC (/μL)	13150 (5100-18600)	12600 (8100-19200)	0.51†
Plt ($\times 10^3$/μL)	487 (239-735)	341 (171-721)	0.13†
PT-INR	1.0 (0.8-1.2)	1.0 (0.9-1.4)	0.21†
F staging	3 (2-4)	3 (1-4)	0.94‡

Data are shown as medians (ranges). †Mann-Whitney U-test, ‡Fisher's exact test.

most SOX9+HepPar1+ cells are likely to be derived from hepatocytes only.

In this study, we demonstrated that progressive cholestatic liver injury after KP leads to decreased SOX9+HepPar1+ cell numbers and increased SOX9+HepPar1- and CK19+ cells numbers (**Table 2** and **Figure 4**), and that SOX9+HepPar1+ cells have a close association with the proliferation of CK19+ cells (**Figure 5**). Several *in vitro* and *in vivo* studies have indicated that hepatocytes and biliary cells have mutual plasticity [17, 21, 25, 26], and have indicated that cells of intermediate phenotype appear at the time of regeneration [17, 21]. A linage tracing study of the relationship between SOX9 and liver regeneration using the 3,5-diethoxycarbonyl-1,4-dihydrochollidine (DDC) injured mouse model demonstrated that some hepatocytes converted to CK19+ cells through cells characterized as Sox9+ epithelial cell adhesion molecule (EpCAM)-negative [21]. Thus, we consider that the SOX9+HepPar1+ cells seen in BA are the cells mediating hepatocyte to CK19+ cell conversion. Although it has generally been considered that terminally differentiated liver epithelial cells do not differentiate into any other phenotypes, recent reports have proposed transdifferentiation, which is part of a regenerative mechanism in some tissues [17, 21, 27]. Transdifferentiation has two proposed models. One is that a cell first dedifferentiates into a progenitor stage before it can differentiate to the new lineage. The other is that a cell directly transdifferentiates to a new phenotype [28]. Several reports suggest that Sox9 functions in the expansion of the pool of multipotent progenitor cells in the development of the pancreas and neural crest [29-31]. Therefore, SOX9 might be involved in dedifferentiation of hepatocytes during the process of transdifferentiation and be involved in expansion of the intermediate cell population between hepatocytes and CK19+ cells.

In contrast to BA, a remarkable expansion of SOX9+HepPar1+ cells and a small number of CK19+ cells was confirmed in AGS (**Figure 6A-D** and Supplement Figure 1). Defective Notch signaling can lead to accumulation of intermediate cells between hepatocytes and biliary cells due to "incomplete" ductular transdifferentiation from hepatocytes [13, 32, 33]. Also, inhibition of Notch signaling led to reducing the level of DR [34]. Thus, Notch signaling may be required for DR progression derived from SOX9+HepPar1+ cells. However, we also consider that Notch signaling is not absolutely essential to induce DR, because CK19+ cells already existed in AGS liver despite insufficiency of Notch signaling (**Figure 6A** and **6B**). Besides, regeneration of peripheral intrahepatic bile duct has been confirmed in adult Albumin-Cre *Rbpj*$^{flox/flox}$ *Hnf6*$^{flox/flox}$ mice, which have with specific deletions of *Rbpj* and *Hnf6* in liver epithelial cells and bile duct insufficiency [22, 35]. *Rbpj* and *Hnf6* are mediators of canonical Notch signaling. Therefore, alterna-

tive signaling pathways other than Notch also have the possibility to contribute to the mechanism of DR.

Several candidates have recently been demonstrated that have capability of biliary transdifferentiation. *In vitro*, TNFα, an inflammatory cytokine secreted by Kupffer cells, promotes biliary transdifferentiation through Jun N-terminal kinase signaling [25], and treatment of liver explants from E10 mice with Wnt3A facilitates biliary differentiation [36]. *In vivo*, excess stimulation of TGFβ signaling induces the appearance of hepatobiliary cells [37, 38]. Therefore, signaling pathways other than Notch might be involved in human hepatocyte conversion or dedifferentiation into SOX9+HepPar1+ cells even differentiation into CK19+ cells.

The level of the SOX9+HepPar1+ ratio might indicate the extent of the progenitor pool, because SOX9 has the function of extending progenitor cells [30]. Thus, we speculated that BA patients with a small SOX9+HepPar1+ cell number at the time of KP could not survive longer with their native liver compared with patients with a large SOX9+HepPar1+ cell number because of insufficient regeneration ability. The cut-off value of the SOX9+HepPar1+ ratio at KP (groups I and II, n=22) determined by ROC analysis was 10.9%. According to this value, the 22 patients were re-divided into two groups; High SOX9+HepPar1+ ratio group (>10.9%, n=5) and Low SOX9+HepPar1+ ratio group (≤10.9%, n=17). The clinical data between native liver survivors and non-native liver survivors showed no statistical differences at KP (**Table 3**), however, cumulative native liver survival rate (P=0.07; Supplement Figure 2) showed that the SOX9+HepPar1+ ratio might be predictive for the prognosis of native liver survival if a greater number of BA patients were recruited.

In conclusion, this is the first study to describe association SOX9+HepPar1+ cells with DR in BA livers. The findings suggest that SOX9+ HepPar1+ cells can have an important role for mediating the progression of DR and that Notch signaling may contribute to DR progression. Future studies for additional elucidation of the mechanism of DR may lead to new therapies for liver regeneration or liver fibrosis induced by cholestatic liver injury.

Acknowledgements

This work was supported by JSPS KAKENHI Grant Number 25462776 to Y.I. We thank Sayoko Fujimura, Liaison Laboratory Promotion Center, Institute of Molecular Embryology and Genetics, Kumamoto University, who processed liver tissues and provided technical assistance.

Disclosure of conflict of interest

None.

Address correspondence to: Dr. Daiki Yoshii, Department of Pediatric Surgery and Transplantation, Graduate School of Medical Sciences, Kumamoto University, 1-1-1 Honjo, Chuo-ku, Kumamoto 860-8556, Japan. Tel: +81-96-373-5616; Fax: +81-96-373-5783; E-mail: d-yoshii@fc.kuh.kumamoto-u.ac.jp

References

[1] Espanol-Suner R, Carpentier R, Van Hul N, Legry V, Achouri Y, Cordi S, Jacquemin P, Lemaigre F and Leclercq IA. Liver progenitor cells yield functional hepatocytes in response to chronic liver injury in mice. Gastroenterology 2012; 143: 1564-1575, e1567.

[2] Popper H, Kent G and Stein R. Ductular cell reaction in the liver in hepatic injury. J Mt Sinai Hosp N Y 1957; 24: 551-556.

[3] Roskams TA, Theise ND, Balabaud C, Bhagat G, Bhathal PS, Bioulac-Sage P, Brunt EM, Crawford JM, Crosby HA, Desmet V, Finegold MJ, Geller SA, Gouw AS, Hytiroglou P, Knisely AS, Kojiro M, Lefkowitch JH, Nakanuma Y, Olynyk JK, Park YN, Portmann B, Saxena R, Scheuer PJ, Strain AJ, Thung SN, Wanless IR and West AB. Nomenclature of the finer branches of the biliary tree: canals, ductules, and ductular reactions in human livers. Hepatology 2004; 39: 1739-1745.

[4] Alvaro D, Mancino MG, Glaser S, Gaudio E, Marzioni M, Francis H and Alpini G. Proliferating cholangiocytes: a neuroendocrine compartment in the diseased liver. Gastroenterology 2007; 132: 415-431.

[5] Glaser SS, Gaudio E, Miller T, Alvaro D and Alpini G. Cholangiocyte proliferation and liver fibrosis. Expert Rev Mol Med 2009; 11: e7.

[6] Desmet V, Roskams T and Van Eyken P. Ductular reaction in the liver. Pathol Res Pract 1995; 191: 513-524.

[7] Roskams T and Desmet V. Ductular reaction and its diagnostic significance. Semin Diagn Pathol 1998; 15: 259-269.

[8] Wagner T, Wirth J, Meyer J, Zabel B, Held M, Zimmer J, Pasantes J, Bricarelli FD, Keutel J, Hustert E, Wolf U, Tommerup N, Schempp W and Scherer G. Autosomal sex reversal and campomelic dysplasia are caused by mutations in and around the SRY-related gene SOX9. Cell 1994; 79: 1111-1120.

[9] Foster JW, Dominguez-Steglich MA, Guioli S, Kwok C, Weller PA, Stevanovic M, Weissenbach J, Mansour S, Young ID, Goodfellow PN, et al. Campomelic dysplasia and autosomal sex reversal caused by mutations in an SRY-related gene. Nature 1994; 372: 525-530.

[10] Antoniou A, Raynaud P, Cordi S, Zong Y, Tronche F, Stanger BZ, Jacquemin P, Pierreux CE, Clotman F and Lemaigre FP. Intrahepatic bile ducts develop according to a new mode of tubulogenesis regulated by the transcription factor SOX9. Gastroenterology 2009; 136: 2325-2333.

[11] Furuyama K, Kawaguchi Y, Akiyama H, Horiguchi M, Kodama S, Kuhara T, Hosokawa S, Elbahrawy A, Soeda T, Koizumi M, Masui T, Kawaguchi M, Takaori K, Doi R, Nishi E, Kakinoki R, Deng JM, Behringer RR, Nakamura T and Uemoto S. Continuous cell supply from a Sox9-expressing progenitor zone in adult liver, exocrine pancreas and intestine. Nat Genet 2011; 43: 34-41.

[12] Hartley JL, Davenport M and Kelly DA. Biliary atresia. Lancet 2009; 374: 1704-1713.

[13] Fabris L, Cadamuro M, Guido M, Spirli C, Fiorotto R, Colledan M, Torre G, Alberti D, Sonzogni A, Okolicsanyi L and Strazzabosco M. Analysis of liver repair mechanisms in Alagille syndrome and biliary atresia reveals a role for notch signaling. Am J Pathol 2007; 171: 641-653.

[14] Suda H, Yoshii D, Yamamura K, Yokouchi Y and Inomata Y. New insight into reactive ductular cells of biliary atresia provided by pathological assessment of SOX9. Pediatr Surg Int 2014; 30: 481-492.

[15] Kodama Y, Hijikata M, Kageyama R, Shimotohno K and Chiba T. The role of notch signaling in the development of intrahepatic bile ducts. Gastroenterology 2004; 127: 1775-1786.

[16] Zong Y, Panikkar A, Xu J, Antoniou A, Raynaud P, Lemaigre F and Stanger BZ. Notch signaling controls liver development by regulating biliary differentiation. Development 2009; 136: 1727-1739.

[17] Yanger K, Zong Y, Maggs LR, Shapira SN, Maddipati R, Aiello NM, Thung SN, Wells RG, Greenbaum LE and Stanger BZ. Robust cellular reprogramming occurs spontaneously during liver regeneration. Genes Dev 2013; 27: 719-724.

[18] Oda T, Elkahloun AG, Pike BL, Okajima K, Krantz ID, Genin A, Piccoli DA, Meltzer PS, Spinner NB, Collins FS and Chandrasekharappa SC. Mutations in the human Jagged1 gene are responsible for Alagille syndrome. Nat Genet 1997; 16: 235-242.

[19] McDaniell R, Warthen DM, Sanchez-Lara PA, Pai A, Krantz ID, Piccoli DA and Spinner NB. NOTCH2 mutations cause Alagille syndrome, a heterogeneous disorder of the notch signaling pathway. Am J Hum Genet 2006; 79: 169-173.

[20] Ichida F, Tsuji T, Omata M, Ichida T, Inoue K, Kamimura T, Yamada G, Hino K, Yokosuka O and Suzuki H. New Inuyama classification; new criteria for histological assessment of chronic hepatitis. International Hepatology Communications 1996; 6: 112-119.

[21] Tanimizu N, Nishikawa Y, Ichinohe N, Akiyama H and Mitaka T. Sry HMG box protein 9-positive (Sox9+) epithelial cell adhesion molecule-negative (EpCAM-) biphenotypic cells derived from hepatocytes are involved in mouse liver regeneration. J Biol Chem 2014; 289: 7589-7598.

[22] Walter TJ, Vanderpool C, Cast AE and Huppert SS. Intrahepatic bile duct regeneration in mice does not require Hnf6 or Notch signaling through Rbpj. Am J Pathol 2014; 184: 1479-1488.

[23] Malato Y, Naqvi S, Schurmann N, Ng R, Wang B, Zape J, Kay MA, Grimm D and Willenbring H. Fate tracing of mature hepatocytes in mouse liver homeostasis and regeneration. J Clin Invest 2011; 121: 4850-4860.

[24] Tarlow BD, Finegold MJ and Grompe M. Clonal tracing of Sox9+ liver progenitors in mouse oval cell injury. Hepatology 2014; 60: 278-289.

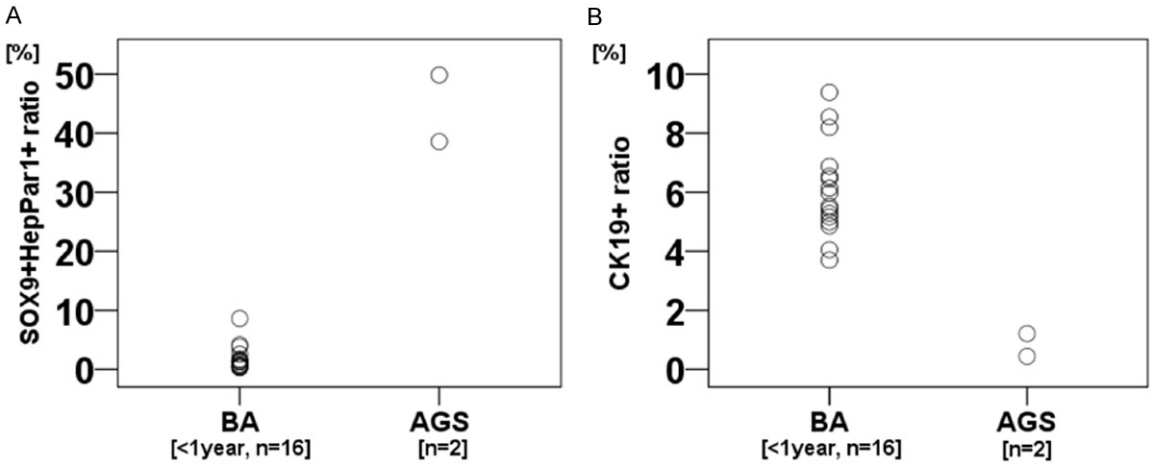
[25] Nishikawa Y, Sone M, Nagahama Y, Kumagai E, Doi Y, Omori Y, Yoshioka T, Tokairin T, Yoshida M, Yamamoto Y, Ito A, Sugiyama T and Enomoto K. Tumor necrosis factor-alpha promotes bile ductular transdifferentiation of mature rat hepatocytes in vitro. J Cell Biochem 2013; 114: 831-843.

[26] Sone M, Nishikawa Y, Nagahama Y, Kumagai E, Doi Y, Omori Y, Yoshioka T, Tokairin T, Yoshida M, Sugiyama T and Enomoto K. Recovery of mature hepatocytic phenotype following bile ductular transdifferentiation of rat hepatocytes in vitro. Am J Pathol 2012; 181: 2094-2104.

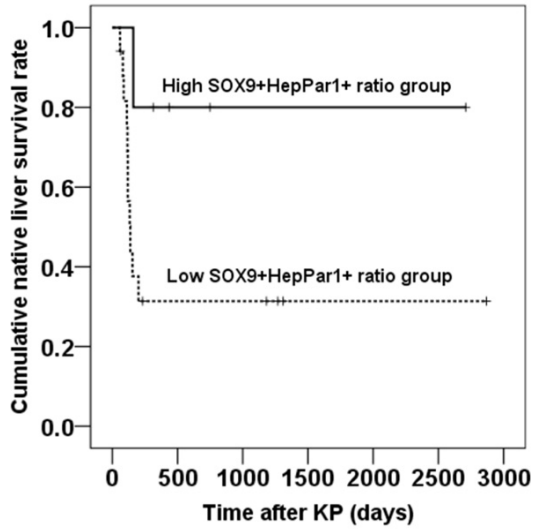
[27] Tsonis PA, Madhavan M, Tancous EE and Del Rio-Tsonis K. A newt's eye view of lens regeneration. Int J Dev Biol 2004; 48: 975-980.

[28] Jopling C, Boue S and Izpisua Belmonte JC. Dedifferentiation, transdifferentiation and reprogramming: three routes to regeneration. Nat Rev Mol Cell Biol 2011; 12: 79-89.

[29] Spokony RF, Aoki Y, Saint-Germain N, Magner-Fink E and Saint-Jeannet JP. The transcription factor Sox9 is required for cranial neural crest development in Xenopus. Development 2002; 129: 421-432.
[30] Seymour PA, Freude KK, Tran MN, Mayes EE, Jensen J, Kist R, Scherer G and Sander M. SOX9 is required for maintenance of the pancreatic progenitor cell pool. Proc Natl Acad Sci U S A 2007; 104: 1865-1870.
[31] Kawaguchi Y. Sox9 and programming of liver and pancreatic progenitors. J Clin Invest 2013; 123: 1881-1886.
[32] Libbrecht L, Spinner NB, Moore EC, Cassiman D, Van Damme-Lombaerts R and Roskams T. Peripheral bile duct paucity and cholestasis in the liver of a patient with Alagille syndrome: further evidence supporting a lack of postnatal bile duct branching and elongation. Am J Surg Pathol 2005; 29: 820-826.
[33] Desmet VJ. Ductal plates in hepatic ductular reactions. Hypothesis and implications. III. Implications for liver pathology. Virchows Arch 2011; 458: 271-279.
[34] Fiorotto R, Raizner A, Morell CM, Torsello B, Scirpo R, Fabris L, Spirli C and Strazzabosco M. Notch signaling regulates tubular morphogenesis during repair from biliary damage in mice. J Hepatol 2013; 59: 124-130.
[35] Vanderpool C, Sparks EE, Huppert KA, Gannon M, Means AL and Huppert SS. Genetic interactions between hepatocyte nuclear factor-6 and Notch signaling regulate mouse intrahepatic bile duct development in vivo. Hepatology 2012; 55: 233-243.
[36] Hussain SZ, Sneddon T, Tan X, Micsenyi A, Michalopoulos GK and Monga SP. Wnt impacts growth and differentiation in ex vivo liver development. Exp Cell Res 2004; 292: 157-169.
[37] Clotman F, Jacquemin P, Plumb-Rudewiez N, Pierreux CE, Van der Smissen P, Dietz HC, Courtoy PJ, Rousseau GG and Lemaigre FP. Control of liver cell fate decision by a gradient of TGF beta signaling modulated by Onecut transcription factors. Genes Dev 2005; 19: 1849-1854.
[38] Raynaud P, Carpentier R, Antoniou A and Lemaigre FP. Biliary differentiation and bile duct morphogenesis in development and disease. Int J Biochem Cell Biol 2011; 43: 245-256.



Supplement Figure 1. Comparison of SOX9+HepPar1+ ratio between BA and AGS. BA patients included all the patients under 1 year old at the time of LT.



Supplement Figure 2. Cumulative native liver survival rate of BA patients (n=22) after KP. Comparison between High SOX9+HepPar1+ ratio group (>10.9%, n=5) and low SOX9+Heppar1+ ratio group (≤10.9%, n=17). Long-rank test, P=0.07.