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Roles of myofibroblasts and Notch and Hedgehog signalling pathways in formation of intrahepatic bile duct lesions of the polycystic kidney rat

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Short title: Notch signalling pathway in PCK rats

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Abstract

Objectives: PCK rats, an animal model of Caroli's disease, show a dilatation of intrahepatic bile ducts (IHBD) called "ductal plate malformation". Mesenchymal cells and the Notch and Hedgehog signalling pathways in portal tracts are reportedly involved in the normal development of IHBD, though there have been no studies on the roles of these signalling pathways in PCK rats. Methods: We examined mainly immunohistochemically the expression of the molecules related to these signalling pathways in portal tracts. Results: All molecules related to these signalling pathways expressed in portal tracts in SD rats (control), were also expressed in PCK rats. Mesenchymal cells (myofibroblasts) were frequently found in the connective tissue of portal tracts of SD and PCK rats at E20D, 1D and 1W, and were abundant in PCK rats. Interestingly, myofibroblasts almost disappeared at 3W in both strains. Jagged1 was expressed in mesenchymal cells in portal tracts, and was abundant in PCK rats. Double immunostaining showed that Jagged1-positive cells were myofibroblasts. Notch2 and HES1 were expressed in cholangiocytes of the bile ducts of both rats. Sonic Hedgehog was expressed in the bile ducts of both rats, similarly. Conclusions: A well-balanced and time-sequential expression of the Notch and Hedgehog family in portal tracts might be essential for the normal development of IHBD from E20D until 1W in SD rats, and an imbalanced interaction of these molecules, particularly increased Jagged1 expression in periductal and periportal myofibroblasts and Notch2 expressed in cholangiocytes, may be involved in the formation of bile duct lesions in PCK rats.

Key words: intrahepatic bile duct, Notch family, myofibroblasts, ductal plate malformation, Caroli's disease

Introduction

Caroli's disease is characterized by the progressive multiple dilatation of intrahepatic bile ducts (IHBD) with congenital hepatic fibrosis (CHF) (1). Most cases of Caroli's disease with CHF show an autosomal recessive inheritance and are associated with autosomal recessive polycystic kidney disease (ARPKD) (2). Polycystic kidney (PCK) rats, an animal model of ARPKD (2,3), spontaneously develop cystic changes of renal tubules and show a multifocal and irregular dilation of IHBD known as “ductal plate malformation (DPM)” and fibrosis of the portal area (2,3). DPM, an abnormal biliary and stromal malformation, is characterized by an irregularly shaped and variably dilated duct lumen covered by a low columnar or cuboidal biliary epithelium, bulging or irregular protrusions covered by biliary epithelium into the dilated lumen, and bridge-like structures covered by biliary epithelium in the dilated lumen (4,5), and DPM is characteristically found in patients with Caroli's disease and CHF or in PCK rats (2-5). ARPKD develops as a result of mutations in the causal *PKHD1* gene. PCK rats show a deficiency of the *pkdh1* protein product, fibrocystin, in the primary cilia, with malformation of primary cilia, which is believed to cause bile duct lesions (6). In addition, the following mechanisms have been also reported to contribute to DPM : disordered cell kinetics of biliary epithelial cells such as the activation of the MEK5-ERK5 cascade (3,7). Basal laminar components and stromal fibrogenesis may also play important roles. Biliary overexpression of plasminogen and tissue-type plasminogen activator leads to the generation of excessive amounts of plasmin, and subsequent plasmin-dependent lysis of the extracellular matrix molecules may contribute to the formation of DPM (8,9). Several genetic changes involving hepatocyte nuclear factor (HNF)-6, HNF 1 β , and cystin were also reported to be involved in the DPM process (8).

The PCK rat is also characterized by progressive hepatic fibrosis (2). Our previous study revealed two different types of intrahepatic bile ducts: those lined with cuboidal-shaped (C-type) and flat-shaped (F-type) cholangiocytes (11). While the latter was thought to participate in progressive hepatic fibrosis by producing extracellular matrix molecules, the exact mechanisms remain speculative.

Notch signalling is an important pathway in the development of various organs, as well as nerves and blood vessels (12,13). In mammals, Notch1 and Notch 2 transduce signals from their ligands, Jagged1 and Jagged 2, expressed on neighboring cells. This Notch pathway is also important for the normal development

of intrahepatic bile ducts (IHBD) (14,15), and anomalous biliary development may be directly or indirectly associated with impaired signalling pathways of the Notch family. For example, mutations in *Jagged1* cause Alagille syndrome (AGS) characterized by a paucity of bile ducts (16-19). *Jagged1* is crucial for the commitment of hepatoblasts to cholangiocytes and also essential for the morphogenesis of IHBD, particularly for the control of the second layer of ductal plates during the remodeling of bile ducts (14). Notch2 signalling pathways also reportedly regulate the differentiation and survival of cholangiocytes and induction of tubulogenesis during the development of IHBD. For example, Tchorz et al reported that expression of the intracellular domain of Notch2 (Notch 2ICD) caused hepatoblasts to differentiate into cholangiocytes, which form additional bile ducts in periportal regions and ectopic ducts in lobular regions, and additional bile ducts in periportal regions are maintained into adulthood, resulting in an increased number of bile ducts per portal tract (15).

It has been reported that mesenchymal cells in the portal area of mice express *Jagged1* and such mesenchymal cells are known to express α -smooth muscle antigen (α -SMA) (14). In mice whose expression of *Jagged1* in portal mesenchymal cells in the portal area has been selectively knocked out, the formation of the tubular structure of the bile ducts is inhibited (17). Taken together, a well-balanced expression and signalling pathway of *Jagged1* in periductal mesenchymal cells and Notch2 in cholangiocytes of IHBD might be mandatory for the normal development of bile ducts (14).

So far, there have been many reports on the pathophysiological roles of Notch family signalling pathways in genetic and acquired biliary diseases such as AGS (16-18). For Caroli's disease, however, there have been few studies on the altered signalling pathways of Hedgehog (Hh) responsible for DPM, and no studies on Notch family signalling pathways in either the disease itself or its animal model, PCK rats (20,21).

In this study, we examined the roles of Notch and Hedgehog signalling pathways mainly by immunostaining molecules related to these pathways in the normal development of IHBD and also the formation of bile duct lesions in PCK rats.

Materials and methods

Animals and collection of liver tissue specimens

PCK rats were maintained at the Laboratory Animal Institute of Kanazawa University Graduate School of Medicine. Normal (Crl:CD(SD)) rats used as a control were purchased from Charles River Japan (Sagamihara, Japan). Livers were removed from fetal (18 and 20 days of gestation), neonatal, and adult (1-day, one-week and 3-week old) rats. After preliminary experiments, more than 5 SD and PCK rats of each age were sacrificed. Liver samples were immersed in a 10% formalin neutral buffer solution (pH 7.4) and then embedded in paraffin, and also freshly embedded in OTC compound and quickly frozen in liquid nitrogen. More than 20 serial sections, 4 μ m thick, were cut from the paraffin-embedded and frozen sections. Several of these sections were stained with hematoxylin and eosin (H&E) and Azan-Mallory, and the remainder were subjected to an immunohistochemical analysis. Parts of the tissue were immediately frozen in liquid nitrogen for use in the reverse transcription-polymerase chain reaction (RT-PCR). In this study, IHBDs and bile ductules were mainly examined. Epithelial cells lining bile ductules and bile ducts are known as cholangiocytes. All animal studies were performed in accordance with the Guidelines for the Care and Use of Laboratory Animals at Takara-machi Campus of Kanazawa University, Kanazawa, Japan.

Immunohistochemistry

Immunostaining of Jagged 1, Jagged 2, Notch 1, Notch 2, Hes1, α -smooth muscle actin (α -SMA), Sonic Hedgehog (Shh), Ki-67 and ssDNA was performed using fresh frozen sections or paraffin-embedded sections of livers from PCK rats and SD rats. Primary antibodies and their sources are shown in Table 1. Frozen sections were fixed with cold acetone followed by blocking of endogenous peroxidase, and incubated with primary antibodies (Jagged 1, Jagged 2, Notch 1, Notch 2, and Hes1) for 1-2h at room temperature. Paraffin-embedded sections were deparaffinized and after blocking of endogenous peroxidase, were incubated with individual primary antibodies (α -smooth muscle actin (α -SMA), Sonic Hedgehog (Shh), Ki-67 and ssDNA) for 1-2 hours at room temperature. Then, the sections were incubated with a secondary antibody conjugated to the peroxidase-labeled polymer, Histofine® Simple Stain Rat MAX PO (Nichirei Biosciences Inc., Tokyo, Japan). Color development was performed using 3,3-diaminobenzidine tetrahydrochloride. The sections for Jagged 1, Jagged 2, Notch 1, Notch 2, α -smooth muscle actin (α -SMA), and Sonic Hedgehog (Shh) were

counter-stained with hematoxylin, lightly. Those for Hes1, Ki-67 and ssDNA were not counterstained. The negative control was evaluated by substitution of the primary antibodies with corresponding non-immunized serum.

Double fluorescence immunostaining

Single-color fluorescence immunostaining of Notch 2, and double-color immunostaining of α -SMA and Jagged 1, α -SMA and Jagged 2, and α -SMA and Notch 1 were performed as follows. Fresh frozen sections were fixed with cold acetone and after the blocking of endogenous peroxidase, incubated overnight at 4°C with antibody for Notch 2 or a mixture of antibodies for α -SMA and Jagged 1 or α -SMA and Jagged 2. The primary antibodies are listed in Table 1. The detection of Notch 2, Jagged 1, and Jagged 2 was performed using a TSA (Tyramide Signal Amplification)^{TM} kit with Alexa Fluor® 488 (Invitrogen, Carlsbad, CA). α -SMA was detected with Alexa Fluor® 594 secondary antibody (Invitrogen). Nuclei were stained with 4', 6-diamidino-2-phenylindole (DAPI).

RT-PCR

Total RNA was extracted from cryostat sections of the liver using an RNA extraction kit (RNeasy Mini, Qiagen K. K., Tokyo, Japan) and used to synthesize cDNA with reverse transcriptase (ReverTra Ace, Toyobo Co., Osaka, Japan). Then PCR was performed with KOD DNA polymerase (KOD-Plus or KOD-Plus-Neo, Toyobo Co.) and primer sets for *Notch1*, *Notch2*, *Jagged1*, *Jagged2*, *HES1*, *Shh*, *Gli1*, and *β -actin*. The sequences of the primers used for PCR are shown in Table 2. The PCR products were subjected to 1% agarose gel electrophoresis and stained with ethidium bromide.

Image analysis

ImageJ 1.45s (National Institutes of Health, USA) was used to measure α -SMA-positive areas in the liver specimens and for gel densitometry for semi-quantitative RT-PCR.

Labeling index of Ki-67 and ssDNA: Ki-67 expression and ssDNA expression were evaluated in more than 500 cholangiocytes of intrahepatic bile ducts in SD and PCK rats, and the percentage of positive cells was expressed as the Ki-67 LI and ssDNA LI. The former reflects proliferative activities and the latter, apoptosis. The data are shown as the mean of 3 separate measurements, and the error bars show standard deviations. *, p<0.05 in Student's t-test or Welch's t-test.

Statistics

The mean and SD was calculated for all parameters. Statistical differences between SD rats and PCK rats were determined using the Student's t-test or Aspin-Welch test, after an analysis of the homogeneity of variance by the F-test. Values of $P < 0.05$ were considered to be statistically significant.

Results

Pathology of liver and bile ducts in PCK rats

PCK rats showed abnormal biliary and stromal changes (DPM) characterized by an irregularly shaped and variably dilated lumen covered by low columnar or cuboidal cholangiocytes (1). These DPM patterns were not clearly identifiable at E18D, but became evident at E20D, 1D, 1W and thereafter in PCK rats (**Fig.1A,B**). Portal tracts were composed of loose connective tissue containing many mesenchymal cells. The loose fibrous stroma was abundant in PCK rats, particularly around the bile ducts and portal veins, in comparison with SD rats. At 3W, such tissue was significantly less abundant and more hypocellular in both rats. In PCK rats, IHBD was largely classifiable into two types at 3W and thereafter: bile ducts lined with cuboidal/columnar cholangiocytes (C-type) and those lined with squamous epithelium similar to mesenchymal cells (F-type) (**Fig.2A**) (11).

Distribution of α -SMA-positive mesenchymal cells (myofibroblasts) in the portal tracts

α -SMA was constantly expressed in the vascular smooth muscle stages before and after birth in both rats. In SD rats, mesenchymal cells positive for α -SMA (myofibroblasts) were also found in the enlarged portal tracts from E20D, 1D and 1W (**Fig.1C, Fig.3A**). At 3W, there were almost no myofibroblasts in the portal tracts. In PCK rats, myofibroblasts were found in the enlarged portal tracts until 1W, as seen in SD rats. Myofibroblasts in the portal tracts were more abundant along with the increased amount of loose connective tissue in PCK rats in comparison with SD rats (**Fig.1D, Fig.3B**). Interestingly, at 3W and later, there were no or few myofibroblasts around C-type bile ducts, similar to SD rats, though myofibroblasts were still present around F-type bile ducts (**Fig. 2B**).

Overall assessment of α -SMA-positive areas: Quantitative analysis of α -SMA-positive areas in the livers showed that the percentage of α -SMA-positive areas increased slightly from E18D to 1D and decreased

thereafter in SD rats (**Fig.3C**). In PCK rats, the percentage of α -SMA-positive areas was higher than in SD rats after E20D and peaked ($5.51 \pm 2.41\%$) at 1W, being 2.7 times higher than in SD rats ($2.00 \pm 0.97\%$) ($p < 0.05$). Later, α -SMA-positive areas decreased and there was no differences between PCK and SD rats at 3W ($1.27 \pm 0.67\%$ and $0.98 \pm 0.52\%$, respectively).

Immunohistochemistry: Expression of Notch family-related factors

Jagged1, Jagged2 and Notch 1: Jagged1 was expressed in the cytoplasm of mesenchymal cells of the portal tracts, particularly around the bile ducts and portal vasculature including portal veins and hepatic arteries at E20D and 1D and in periportal areas and focally around the portal vasculature at 1W in both SD and PCK rats (**Fig.4A,B**). Double immunostaining showed that a majority of Jagged1-positive cells were also positive for α -SMA. Jagged1+ mesenchymal cells in portal tracts of PCK rats outnumbered those of SD rats at E20D, 1D and 1W. At 3W, Jagged1 was not expressed in mesenchymal cells in the portal tracts of SD and PCK rats, except for its expression on scattered mesenchymal cells around C-type bile ducts.

Jagged2 and Notch1 were also expressed in the cytoplasm of mesenchymal cells scattered in the portal tracts, particularly around the portal veins at E20D, 1D and 1W in PCK and SD rats (**Fig.5A,B**). Double immunostaining showed that all Jagged2 and Notch1-positive cells were also positive for α -SMA. Jagged2+ and Notch 1+ mesenchymal cells were not found in the portal tracts after 3W in either strain except on a few mesenchymal cells around F-type bile ducts.

Notch2: Notch2 was diffusely expressed in the cytoplasm of hepatocytes and cholangiocytes of both rats, and its expression was rather weak in the hepatocytes (**Fig.6A,B**). The expression of cytoplasmic Notch2 was similar in cholangiocytes at E20D, 1D, 1W and 3W in both rats. It was also similarly positive in cholangiocytes of F and C type rats. It was focally expressed in mesenchymal cells of the portal tracts.

Hes1: Hes1 was expressed in the nuclei of cholangiocytes of the bile ducts throughout the developmental stages examined, and its expression was slightly stronger in PCK rats in comparison with SD rats. It was expressed similarly in F type and C type rats (**Table 3**).

RT-PCR: mRNA level of Notch family-related factors

The time-course of mRNA expression of *Notch1*, *Notch2*, *Jagged1*, *Jagged2* and *Hes1* in liver specimens of both rats is shown in **Fig.7A-E**. During the fetal period through the age of 3W, the mRNA level of *Notch1* in

PCK rats was similar to that in SD rats. Meanwhile, the level of mRNA of *Notch2*, *Jagged1*, and *Jagged2* was similar at E18D and E20D in PCK rats and SD rats, but tended to be higher in PCK rats from D1 and/or 1W.

Expression of Hedgehog family-related factors

Immunohistochemical expression of Shh: Shh was weakly expressed in the cytoplasm of cholangiocytes in PCK and SD rats (**Fig 8 A,B**). It was expressed similarly in cholangiocytes of F type and C type bile ducts of PCK rats.

mRNA level of Shh: RT-PCR showed that the expression of Shh mRNA was very low or undetectable at E18D, E21D, and D1, but increased at 3W in both PCK and SD rats, with no statistically significant difference between the two strains (**Fig 8 C**).

Expression of Ki67 and ssDNA

The Ki67 and ssDNA LIs of cholangiocytes of the bile ducts are shown in **Fig. 9**. While the Ki-67 LI was $6.1 \pm 7.2\%$, $7.4 \pm 4.4\%$ and $9.5 \pm 5.7\%$ in SD rats at 1D, 1W and 3W, it was higher significantly in PCK rats ($8.9 \pm 3.9\%$, $15.3 \pm 5.6\%$ and $17.7 \pm 4.3\%$). In contrast, the ssDNA LI was lower, and there was no difference at 1D, 1W and 3W between PCK and SD rats.

Discussion

The findings of this study can be summarized as follows: i) α -SMA+ mesenchymal cells (myofibroblasts) were diffusely distributed in portal tracts of both rat strains while they were abundant in PCK rats. Interestingly, such cells almost disappeared at 3W in both strains except around the F-type bile ducts of PCK rats. ii) Jagged1 was expressed in myofibroblasts in portal tracts, particularly around the bile ducts and portal vasculature at E20D and 1D and in periportal areas at 1W in both rats, and Jagged1+ myofibroblasts were rather abundant in PCK rats. Notch2 was expressed in the cholangiocytes in both strains. iii). Shh was similarly expressed in the cholangiocytes of bile ducts in both rats. iv) All the Notch and Hedgehog signalling pathway molecules examined were expressed in myofibroblasts and/or cholangiocytes of both rats. v) A well-balanced expression of Notch and Hedgehog-related molecules in portal tracts is important for the normal development of IHBD in SD rats, and an imbalanced expression of Jagged1 in periductal and

periportal myofibroblasts and interaction with Notch2 on the cholangiocytes may be involved in the formation of bile duct lesions in PCK rats.

Previous studies suggested that cells or factors in the portal areas contributed to the differentiation of hepatoblasts into cholangiocytes and formation of bile ducts (22). IHBD is formed along with the portal veins consisting of endothelial cells and mesenchymal cells, and at the later gestational stage, the interaction of myofibroblasts and cholangiocytes reportedly contributes to the formation of IHBD in mice (14). It was found in this study that the myofibroblasts were distributed in the portal tracts from E20D until 1W in SD rats, suggesting that the interaction of portal myofibroblasts and cholangiocytes at this stage is important in the normal development of IHBD. There are several reports that Jagged1+ mesenchymal cells located in the portal area, particularly in the periportal area where ductal plates and bile ducts are formed and remodeled, were important for the development of IHBD (14). Jagged1+ portal vein mesenchymal cells instruct hepatoblasts expressing Notch2, a receptor of Jagged1, to become cholangiocytes and hence induce the differentiation of hepatoblasts into cholangiocytes (14,23). The signalling pathway of Notch2 in cholangiocytes promotes also fate specification of bile ducts and tubulogenesis in mice (24-26). A hallmark of Notch signalling pathways is the requirement of direct cell-to-cell contact, as both Notch ligands and receptors are integral membrane proteins (27). Thus, IHBD development in the late gestational stage is dependent on Jagged1 and Notch2 expressed in portal tracts (14,16-18). This study also showed that Jagged1 was expressed in periductal and periportal myofibroblasts and Notch2 was expressed in cholangiocytes of SD rats at this stage (E20D - 1W), supporting that a well-balanced and time sequential expression of Jagged1 and Notch2 in the portal tracts and their interaction are essential in the development of IHBD.

Impaired signalling pathways of the Notch family, particularly Notch2 and Jagged1, may be therefore involved in the development of anomalous IHBD (16-18). For example, loss of Jagged1 expression in the periductal mesenchyma due to mutations of *Jagged1* leads to AGA with defective bile duct development beyond the initial formation of the ductal plate (20). In contrast, activation of the Notch pathway by forced expression of the Notch intracellular domain in hepatoblasts enhanced cholangiocyte differentiation but suppressed hepatocyte differentiation (14,23). The sustained expression of the intracellular domain of Notch2 (Notch 2ICD) led to additional bile ducts in periportal regions and ectopic ducts in lobular regions, and the

additional bile ducts in periportal regions were maintained into adulthood, resulting in an increased number of bile ducts per portal tract (15).

PCK rats are known to show DPM (failure of remodeling of bile ducts), and several pathogenetic mechanisms such as altered cell kinetics have been proposed for the DPM (2,3,11,21,4,21). However, there have been no studies on the roles of Notch and Hedgehog expression and signalling pathways in cholangiocytes and mesenchymal cells in the livers of PCK rats. It was found in this study that myofibroblasts were abundant in PCK rats in comparison with SD rats at E20D, 1D and 1W, and this abundance was also confirmed by semiquantitative evaluation of α -SMA immunostaining. This study showed that the Notch family members examined were expressed in the liver of PCK rats as seen in control rats. However, the number of Jagged1+ myofibroblasts was apparently increased around the bile ducts and portal vasculature and also in the periportal area of PCK rats. Thus, the cholangiocytes of bile ducts of PCK rats at this stage were speculated to be exposed to increased and continuous Jagged1 expression from myofibroblasts. Notch2 expressed in cholangiocytes of bile ducts may interact with Jagged1, suggesting that the myofibroblast-mediated continuous and enhanced activation of Notch 2 - Jagged1 interaction in cholangiocytes might contribute to the bile duct lesions of PCK rats. RT-PCR also showed that the mRNA expression of *Notch2* and *Jagged1* was up-regulated in PCK rats after birth, supporting the above-mentioned suggestion. The finding that the Ki-67 LI was high in the cholangiocytes of PCK rats, suggested that increased proliferation of cholangiocytes due to the activation of the Notch2/Jagged1 signalling pathway might have contributed to the formation of DPM (2). Taken together, imbalanced interaction of Jagged1 from myofibroblasts with Notch2 in cholangiocytes may be involved in the formation of bile duct lesions in PCK rats. In contrast, Jagged2 was mainly expressed in myofibroblasts around the portal veins, suggesting that Jagged2 was not involved directly in the bile duct lesions of PCK rats at this developmental stage.

Hes1, a downstream target of Notch signalling pathways and regulator of bile duct morphogenesis (24,25), was expressed in the nuclei of cholangiocytes of IHBD at E20D, 1D and 1W, and its expression on cholangiocytes was slightly stronger and its mRNA level was increased in PCK in comparison with SD rats. Interaction of Notch2 in cholangiocytes with Jagged1+ myofibroblasts may activate Hes1 in cholangiocytes, possibly involved in the formation of DPM. Meckel syndrome (MKS) is an another rare autosomal recessive

disease with DPM and polycystic kidneys (20,21). The gene mutated in type 1 MKS (*Mks1*) encodes a protein associated with the cilium in vertebrates, and loss of function of mouse *Mks1* is an animal model of human MKS with DPM. This bile duct alteration is likely to be the result of the disruption of the Hh signalling pathway. However, the present study showed that *Shh* was similarly expressed in bile ducts and the mRNA level of *Shh* was similar in the livers of both rats, suggesting that disruption of the *Shh* signalling pathway was unlikely involved in the formation of DPM in PCK rats.

Liu et al reported that in the lung and kidney, *Notch1* after interaction with its receptor, *Jagged1*, caused the activation of myofibroblasts (26). *Notch1/Jagged1* interaction may also contribute to the enlargement of mesenchymal cells in the portal area during the formation of bile ducts (19). It was found in this study that like *Jagged1*, *Notch1* was expressed in mesenchymal cells in the portal tracts, particularly around the bile ducts and portal veins, in PCK and SD rats and that numbers of *Notch1*-positive myofibroblasts were increased as seen for *Jagged1* in PCK rats in comparison with SD rats, suggesting that mutual interaction of *Jagged1* and *Notch1* in myofibroblasts, might have resulted in an increased number of myofibroblasts themselves around bile ducts.

As for the two different types of bile ducts in PCK rats (11), there were no α -SMA-positive myofibroblasts around C-type bile ducts, but F-type bile ducts remained surrounded by myofibroblasts expressing *Jagged1* and *Notch1*. According to our previous study (11), F-type bile ducts showed reduced immunohistochemical expression of the biliary epithelial marker CK 19 and aberrant immunoreactivity for vimentin and fibronectin. In mouse cholangiocytes co-cultured with myofibroblastic hepatic stellate cells, Hh ligands from these stellate cells promoted EMT of cholangiocytes. The activation of Hedgehog signalling pathways induces immature cholangiocytes to express mesenchymal markers in the bile ducts and also to undergo epithelial mesenchymal transition (EMT), which are likely to contribute to bile duct lesions and fibrosis (27). It was found in this study that *Shh* was expressed in the cholangiocytes of the F-type and C type bile ducts in PCK rats. *Shh* expressed in F-type bile ducts may be involved in the EMT of these cholangiocytes, suggesting Hedgehog signalling pathways to be at least partly involved in the EMT of F-type bile ducts of PCK rats.

In conclusion, the results obtained here suggest that all members of the Notch and Hedgehog families examined were expressed in portal tracts and bile ducts of both PCK and SD rats and that number of Jagged1+ myofibroblasts were increased around the bile ducts of PCK rats. A relatively increased level of Jagged1 expression in myofibroblasts along with Notch2 expression in cholangiocytes may be involved in the formation of DPM in PCK rats. More studies are needed to test whether these molecules related to Notch signalling pathways could be a target in the treatment of Caroli's disease.

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Figure legends

Fig.1. Bile duct lesions of PCK and SD rats (1 day-old). A: There was connective tissue where bile ducts were located. Portal area of SD rats. B: Dilatation of the ductal plate was evident and there was loose and cellular connective tissue in the portal tract, including around the bile ducts, in the PCK liver. BD, bile duct; PV, portal vein. Bar = 100 μ m. H&E.

Fig. 2. Phenotypes of intrahepatic bile ducts in PCK rats (3 weeks old) and distribution of myofibroblasts. A: Two types of intrahepatic bile duct, those lined by flat-shaped (F-type) cholangiocytes and by cuboidal-shaped (C-type) cholangiocytes, were found in 3-week-old PCK rats. Bar = 100 μ m. HE. B: α -smooth muscle actin (α -SMA)-positive myofibroblasts were distributed around the F-type bile ducts but not around C-type bile ducts and in other parts of portal tracts. Bar = 100 μ m. Immunohistochemical staining for α -SMA counterstained by hematoxylin.

Fig 3. Immunohistochemical staining for α -smooth muscle actin (α -SMA) (red) in the liver of PCK rats and SD rats. **A,B:** Expression of α -SMA in portal areas through the development of bile ducts in SD rats (A) and PCK rats (B). In SD rats (A), α -SMA-positive mesenchymal cells (myofibroblasts) were found in the portal tracts, particularly around the bile ducts and portal veins, from embryonic day 20 (E20D) until postnatal week 1 (1W). Later, at 3 weeks after birth (3W), vascular smooth muscles remained present, though the expression of α -SMA in mesenchymal cells in portal tracts including periductal areas disappeared. In PCK rats (B), until 1 week after birth, the distribution of α -SMA expression was similar to that in SD rats. However, α -SMA-positive regions were abundant in comparison with those in SD rats. In addition, in PCK rats, F-type bile ducts (arrow) at 3 weeks after birth were still surrounded by α -SMA-positive cells. Bar = 100 μ m. **C:** Comparison of α -SMA-positive areas in the liver specimens between SD and PCK rats (percentage of positive areas) according to age showed that α -SMA-positive areas of PCK rats (●) were abundant in comparison with SD rats (○); PCK rats showed a peak at 1w after birth. However, there was a significant difference at 1W ($p < 0.05$). The data are shown as the mean of 5 separate measurements, and the error bars show standard deviations.

Fig 4. Double immunofluorescence staining for Jagged1 (green) and α -smooth muscle actin (α -SMA) (red) in SD rats (A) and PCK rats (B) at 1day and 1week of age.

A: Expression of Jagged1 was found in mesenchymal cells of the portal tracts, particularly around portal veins and also bile ducts at 1D and in periportal areas and focally around bile ducts and blood vessels at 1W in SD rats. A majority of Jagged1-positive cells were also positive for α -SMA. **B:** Jagged1 was expressed in mesenchymal cells of the portal tracts, particularly around blood vessels and also bile ducts at 1D and in periportal areas and focally around blood vessels at 1W in PCK rats. A majority of Jagged1-positive cells were also positive for α -SMA. The number of Jagged1+ cells and α -SMA+ cells of PCK rats outnumbered that of SD rats. BD, bile duct; PV, portal vein. Nuclear staining was performed with DAPI (in blue).

Fig. 5. Double immunofluorescence staining for Jagged2 (green) and α -smooth muscle actin (α -SMA) (red) in SD rats (A) and PCK rats (B) at 1day and 1week of age. **A:** Expression of Jagged2 was mainly found in mesenchymal cells of the portal tracts, particularly around portal veins at 1D and 1W. **B:** Jagged1-positive cells were also distributed in the portal tracts, particularly around the portal vein as seen in SD rats. BD, bile duct; PV, portal vein. Nuclear staining was performed with DAPI (in blue).

Fig. 6. Immunohistochemical staining for Notch2 in the liver of SD rats (A) and PCK rats (B) at 1week of age. Notch2 was weakly expressed in the cytoplasm of hepatocytes and evidently expressed in that of cholangiocytes of both rats. BD, bile duct; PV, portal vein. Bar = 100 μ m.

Fig. 7. Time-course of mRNA expression of *Notch1* (A), *Notch2* (B), *Jagged1* (C), *Jagged2* (D) and *Hes1* (E) measured by RT-PCR in liver specimens of SD ($\circ$) and PCK ($\bullet$) rats. During the fetal period through to the age of 3W, there was no difference in the mRNA level of *Notch1* between PCK rats and SD rats. Meanwhile, while the levels of mRNA for *Notch2*, *Jagged1*, and *Jagged2* were similar at E18D and E20D in PCK and SD rats, they tended to be slightly higher in the PCK rats from D1 and/or 1W. *, <P0.05

Fig. 8. Expression of Sonic Hedgehog in the livers of PCK and SD rats.

A, B: Immunohistochemical staining showed that Shh was expressed similarly in the cytoplasm in bile ducts of SD rats (A) and PCK rats (B) at 1W (bile ducts: arrows).

C: RT-PCR analysis of the mRNA expression of *Shh* in the livers of PCK rats (●) and SD rats (○) from embryonic day 18 until postnatal week 10. The values are shown as ratios with respect to β -actin expression. The mRNA level of *Shh* was similar in SD rats and PCK rats. The data are shown as the mean of 5 separate measurements, and the error bars show standard deviations.

Fig 9. Immunohistochemical study of ki-67 and ssDNA in the bile duct epithelium of PCK rats and SD rats.

A,B: Labeling indexes (LIs) of ki-67 and ssDNA in cholangiocytes of the bile ducts of PCK rats (○) and SD rats (●) are shown. The ki-67 LI was significantly higher in PCK rats (○) than in SD rats (●) (A). Meanwhile, there was no difference in the ssDNA LI between PCK rats and healthy rats. The data are shown as the mean of 5 separate measurements, and the error bars show standard deviations. *, $p < 0.05$ with Student's t-test or Welch's t-test.

Table 1. Primary Antibodies Used for the Immunohistochemical Analysis

Antibody	Species	Supplier	Dilution	Specimen
Jagged1 (H-114)	Rabbit	SCB	1/50	Frozen
Jagged2 (H-143)	Rabbit	SCB	1/50	Frozen
Notch1 (C-20)	Rabbit	SCB	1/50	Frozen
Notch2 (LS-B719)	Rabbit	Lifespan	1/50	Frozen
HES1 (H-140)	Rabbit	SCB	1/50	Frozen
Alpha-Smooth muscle actin	Mouse	Dako	1/100	Paraffin (MW)
Sonic Hedgehog (GT15032)	Goat	Neuromics	1/50	Paraffin (MW)
Ki-67	Mouse	Dako	1/100	Paraffin (MW)
ssDNA	Rabbit	Dako	1/100	Paraffin (MW)

SCB, Santa Cruz Biotechnology, Inc. (Santa Cruz, CA); Lifespan, LifeSpan BioSciences, Inc. (Seattle, WA); DAKO, DakoCytomation (Glostrup, Denmark); Neuromics, Neuromics (Edina, MN); MW, Antigen retrieval was performed by microwaving in 10 mmol/L citrate buffer pH 6.0.

Table 2. Sequences of the Primers Used

Gene	Sequences	Product size
Beta-actin	5'-ACCTTCAACACCCCAGCCATGTACG-3'	698
	5'-CTGATCCACATCTGCTGGAAGGTGG-3'	
Jagged1	5'-CCAGAATGGCAACAAAACCT-3'	459
	5'-CAGCCTGGAGAACACTCACA-3'	
Jagged2	5'-AACGACTTCTTCGGCCACTA-3'	465
	5'-AAAGCCAGACAGCACTTCGT-3'	
Notch1	5'-GTTTGTGCAAGGATGGTGTG-3'	482
	5'-GCACTCGTTGACCTCAGACA-3'	
Notch2	5'-GAGTGTCTGAAGGGCTACGC-3'	460
	5'-TGCAGATGCAGGTGTAGGAG-3'	
HES1	5'-GCTGGAGAAGGCAGACATTC-3'	430
	5'-AAAAACCTTGGCAGCCTCTC-3'	
Sonic Hedgehog	5'-CTGGCCAGATGTTTTCTGGT-3'	117
	5'-TAAAGGGGTCAGCTTTTTTGG-3'	

Table 3 Summary of Immunohistochemical expression of Notch1/2 and Jagged1/2 in the liver of the PCK rat

Cell components	Notch1	Notch2	Jagged1	Jagged2
Cholangiocyte	-	++	-	-
Hepatocyte	-	+	-	-
Myofibroblast	++	+	++	++

-, no remarkable; +, slightly expressed; ++, markedly expressed.

Fig.1

A

B

C

D

Fig.2

Fig.3

Fig.4

Fig.5

Fig.6

Fig.7

Fig.8

Fig.9