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Therapeutic Approaches for Biliary Dysgenesis of the PCK Rat, an Animal Model of Caroli's Disease with Congenital Hepatic Fibrosis

Yasunori Sato, Xiang Shan Ren, Shinichi Furubo and Yasuni Nakanuma*

Department of Human Pathology, Kanazawa University Graduate School of Medicine, Japan

Abstract

The polycystic kidney (PCK) rat shows multiple segmental and saccular dilatations of the intrahepatic bile ducts associated with portal fibrosis, and is an orthologous rodent model of Caroli's disease with congenital hepatic fibrosis as well as autosomal recessive polycystic kidney disease. A cholangiocyte cell line that retains properties of the biliary epithelium lining the bile ducts *in vivo* has been developed from the PCK rat, and it has provided a novel *in vitro* system to study the mechanisms of biliary cystogenesis. In particular, the 3-D culture system is useful to explore the effects of therapies on biliary cystogenesis. Studies using the PCK rat and cultured cholangiocytes have revealed that the biliary dysgenesis is associated with cholangiocyte hyperproliferation, cell-matrix interactions and accelerated fluid transport. The levels of cAMP and intracellular calcium in the cholangiocytes are closely associated with the cyst pathogenesis, and several key signaling pathways such as the activation of B-Raf/MEK/ERK signaling pathway have been identified. This article reviews the advances in therapeutic approaches aiming for ameliorating the hepatobiliary lesions of the PCK rat, particularly focusing on those for the biliary cystogenesis.

Keywords: Caroli's disease; Congenital hepatic fibrosis; Polycystic kidney rat; Biliary dysgenesis; Pharmacotherapy

Abbreviations: α-SMA: α-Smooth Muscle Actin; ARPKD: Autosomal Recessive Polycystic Kidney Disease; Camp: Adenosine 3', 5'-cyclic monophosphate; Cdc25A: Cell Division Cycle 25A; CFTR: Cystic Fibrosis Transmembrane Conductance Regulator; CHF: Congenital Hepatic Fibrosis; CK: Cytokeratin; EGFR: Epidermal Growth Factor Receptor; Epac: Exchange Proteins Directly Activated by cAMP; LPS: Lipopolysaccharide; MEK/ERK: Mitogen-Activated Protein Kinase Kinases/Extracellular Signal-Regulated Kinase; mTOR: Mammalian Target of Rapamycin; mTORC: mTOR Complex; PCK: Polycystic Kidney; PI3K: Phosphatidylinositol 3-kinase; PLD: Polycystic Liver Disease; PPARγ: Peroxisome Proliferator Activator Receptor Gamma; siRNA: Small Interfering RNA; SSTR: Somatostatin Receptor; VPV2R: Vasopressin V2 Receptor; Trpv4: Transient Receptor Potential Vanilloid 4; VEGF: Vascular Endothelial Growth Factor

Introduction

Caroli's disease is characterized by progressive cystic dilatation of the intrahepatic bile ducts, and belongs to an entity of fibropolycystic liver disease [1]. It is often accompanied by congenital hepatic fibrosis (CHF), and is also known as a hepatic manifestation of autosomal recessive polycystic kidney disease (ARPKD) [2]. ARPKD has been estimated to have an incidence of 1 in 20,000 live births, and a significant proportion of cases (up to 30%) die by the neonatal period, primarily of respiratory insufficiency. In the survivors, hypertension and renal insufficiency are the major signs of renal disease, and progression to end-stage renal disease occurs in 20 to 45% of cases within 15 years. A proportion of the patients maintain renal function into adulthood, where they typically have less severe kidney disease, and complications of the liver disease become predominant. These complications include those associated with portal hypertension related to CHF, and cholangitis related to Caroli's disease. Treatment for Caroli's disease with CHF is largely supportive, and is directed toward treating these complications.

Biliary dysgenesis in Caroli's disease with CHF is regarded as a consequence of ductal plate malformation, where the disease pathogenesis has not been fully understood [3]. The polycystic kidney (PCK) rat is an orthologous rodent model of Caroli's disease with CHF as well as ARPKD, and the mutation in the PCK rat has been shown to be an exon deletion in the rat homologue of human *PKHD1* [4]. The development of the PCK rat has allowed the molecular pathogenesis of the disease and the therapeutic approaches to be examined [5].

The mechanism responsible for the hepatobiliary and kidney lesions of the PCK rat is currently being analyzed, and attempts have been made aiming for ameliorating the pathological lesions based on their corresponding mechanisms. This article reviews the advances in therapeutic approaches for the hepatobiliary lesions of the PCK rat, particularly focusing on those for the biliary cystogenesis. First, histopathological features of the PCK rat including their mechanical and pathogenic aspects are briefly described.

Histopathology of the PCK Rat

The ductal plates are dilated in the fetal liver of the PCK rat, representing ductal plate malformation (Figure 1A). The ductal dilatation spreads throughout the liver, and the overgrowth portal connective tissue occurs after delivery. Multiple segmental and saccular dilatations of the intrahepatic bile ducts progress with increasing age, and portal fibrosis also progresses (Figure 1B). These histological features of the PCK liver closely resemble those of Caroli's disease with CHF [6].

Primary cilia of the cholangiocytes of the PCK rat show structural and functional abnormalities. Fibrocystin, a protein product of *PKHD1*, is normally localized to primary cilia, while defects in fibrocystin from primary cilia are observed in the PCK cholangiocytes [7]. Ciliopathies

***Corresponding author:** Yasuni Nakanuma, MD, Department of Human Pathology, Kanazawa University Graduate School of Medicine, 13-1 Takara-machi, Kanazawa 920-8640, Japan, Tel: +81-76-265-2195; Fax: +81-76-234-4229; E-mail: nakanuma@staff.kanazawa-u.ac.jp

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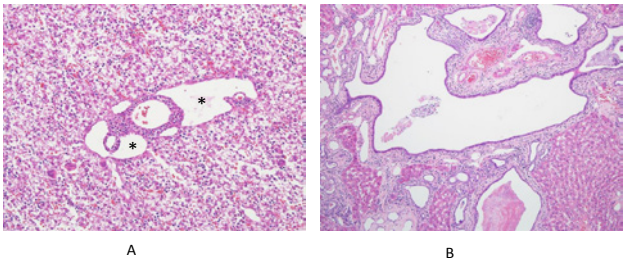

Figure 1: Histopathology of the liver of the PCK rat
A: The ductal plates are dilated in the fetal liver of the PCK rat (*). B: Dilatation of the intrahepatic bile ducts and portal fibrosis of the adult PCK liver. Hematoxylin-eosin staining (A,B).

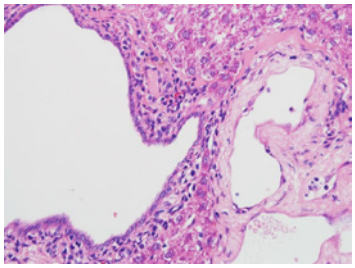

Figure 2: Two different types of intrahepatic bile ducts of the PCK rat
The intrahepatic bile ducts of the PCK rat display two different phenotypes, bile ducts lined by cuboidal-shaped (left) and flat-shaped (right) cholangiocytes. Hematoxylin-eosin staining.

involving the PCK cholangiocytes may result in decreased intracellular calcium and increased adenosine 3', 5'-cyclic monophosphate (cAMP) concentrations, causing cholangiocyte hyperproliferation, abnormal cell-matrix interactions, and altered fluid secretion. These modifications can ultimately result in bile duct dilatation.

Cholangitis arising from biliary infection becomes a frequent histological finding in aged PCK rat, and neovascularization around the bile ducts also increases in aged PCK rat. Lipopolysaccharide (LPS) induces overexpression of vascular endothelial growth factor (VEGF) in the PCK cholangiocytes, which may lead to hypervascularity around the bile ducts [8]. Concurrently, LPS and VEGF act as cell proliferative factors for the cholangiocytes. Thus, biliary infection may thus exacerbate biliary cystogenesis in the PCK rat.

The matrix proteins of the basement membrane of the intrahepatic bile ducts are degraded in the PCK rat. The biliary epithelium of the PCK rat sits the basement membrane which displays abnormal decreases in laminin and type IV collagen expression [9]. The PCK cholangiocytes overexpress plasminogen and tissue-type plasminogen activator, and the resultant generation of excessive amounts of plasmin and the subsequent plasmin-dependent lysis of the extracellular matrix molecules may contribute to the progressive cystic dilatation of the bile ducts.

In most types of chronic liver disease, α-smooth muscle actin (α-SMA)-expressing activated hepatic stellate cells/myofibroblasts play major roles in hepatic fibrosis by producing extracellular matrix molecules. In the PCK liver of the fetus, myofibroblasts are found abundantly in the connective tissue of portal tracts. These myofibroblasts express Jagged1, while the bile duct epithelium expresses Notch2 [10]. An imbalanced interaction of these molecules may be involved in the formation of bile duct lesions of the PCK rat.

Myofibroblasts usually disappear in the portal tracts of the PCK liver around 3 weeks of age, and they are also negligible in hepatic parenchyma in adult PCK liver. Therefore, progressive hepatic fibrogenesis may be mediated by cells other than hepatic stellate cells and portal fibroblasts. Interestingly, the intrahepatic bile ducts of the PCK rat display two different phenotypes, bile ducts lined by cuboidal-shaped and flat-shaped cholangiocytes (Figure 2). The latter usually accompany with dense fibrosis around them. The flat-shaped cholangiocytes immunohistochemically show reduced expression of the biliary epithelial marker cytokeratin (CK)19 and positive expression of the mesenchymal markers such as vimentin and fibronectin, suggesting that cholangiocytes with mesenchymal features contribute to progressive hepatic fibrosis of the PCK rat [11].

In the kidneys of the fetus of PCK rat, cystic dilatation of renal tubules is unremarkable [6]. Small cysts were histologically visible in the neonatal PCK rat at 3 weeks of age, and there are cystic dilatations of renal tubules at the corticomedullary junction and outer layer of medulla. The cystic dilatations spread to the cortex in older animals, and there are no glomerulocystic lesions.

Inhibition of Biliary Cystogenesis of the PCK Cholangiocytes *In Vitro*

A cholangiocyte cell line that retains properties of the biliary epithelium lining the bile ducts *in vivo* has been developed from the PCK rat [12,13]. The PCK cholangiocytes are characterized by a higher rate of proliferation with a doubling time approximately half that of the normal cholangiocytes. The cells express γ-glutamyl transpeptidase and biliary epithelial markers CK7 and CK19. Cilia in the PCK cholangiocytes are short and malformated without the expression of fibrocystin. Notably, the PCK cholangiocytes seeded in 3-D cultures form cystic structures (Figure 3), and the PCK cysts grow more rapidly compared to that of the normal cholangiocytes.

The cholangiocyte cell line has provided a novel *in vitro* system to study the mechanisms of cyst growth and expansion, and is helpful to dissect the differences in cellular response by normal and cystic cholangiocytes to many factors and stimuli involved in cell proliferation, secretion and cell-matrix interactions that underlie the biliary cystogenesis [14]. In particular, the 3-D culture system is useful to explore the effects of therapies on biliary cystogenesis of the PCK rat. Using this system, several studies have investigated the molecular mechanism and therapeutic approaches for the biliary cystogenesis. Therapies reported to be effective for inhibiting cystic growth of the PCK cholangiocytes in the 3-D culture system are summarized in Table 1.

Somatostatin and its analogue, such as octreotide and pasireotide, have been shown to inhibit elevated cAMP levels and to decrease fluid

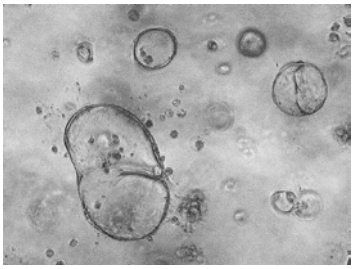

Figure 3: Cysts formed in 3-D culture by the PCK cholangiocytes
The PCK cholangiocytes form cystic structures in Matrigel matrix.

Therapy	Potency	Reference
Octreotide	Somatostatin analogue	[15]
Pasireotide	Somatostatin analogue	[16]
Vitamin K3	Cdc25A inhibitor	[17]
Gefitinib	EGFR tyrosine kinase inhibitor	[18]
4αPDD	Trpv4 activator	[19]
5'6'-EET	Trpv4 activator	[19]
Nifedipine+arachidonic acid	Trpv4 activator	[19]
NPPB	CFTR inhibitor	[20]
CFTRinh172	CFTR inhibitor	[20]
DIDS	Cl-/HCO_3 exchange inhibitor	[20]
SITS	Cl-/HCO_3 exchange inhibitor	[20]
U0126	MEK/ERK inhibitor	[21]
Epac siRNA	Gene silencing	[21]
MicroRNA15a transfection	Overexpression	[22]

4αPDD, 4α-phorbol 12,13-didecanoate; 5'6'-EET, 5'6'-epoxyecosatrienoic acid; Cdc25A, cell division cycle 25A; CFTR, cystic fibrosis transmembrane conductance regulator; DIDS, 4,4'-diisothiocyanatostilbene-2,2'-disulfonic acid disodium salt hydrate; EGFR, epidermal growth factor receptor; Epac, exchange proteins directly activated by cAMP; MEK/ERK, mitogen-activated protein kinasekinases/extracellularsignal-regulatedkinase;NPPB, 5-nitro-2-(3-phenylpropylamino)-benzoic acid; siRNA, small interfering RNA; SITS, 4-acetamido-4'-isothiocyanato-2,2'-stilbenedisulfonic acid disodium salt hydrate; Trpv4, transient receptor potential vanilloid 4.

Table 1: Therapies effective for inhibiting cystic growth of the PCK cholangiocytes in the 3-D culture system.

secretion and cell proliferation in cholangiocytes. Indeed, octreotide and pasireotide inhibit cystic growth of the PCK cholangiocytes in the 3-D culture system, where pasireotide has more prominent effects on the PCK cyst growth that those of octreotide [15,16]. Other example of inhibitor of the PCK cyst growth in the 3-D culture system is vitamin K3. Pharmacologic inhibition of cell division cycle 25A (Cdc25A) with vitamin K3 alters the cell cycle and reduces proliferation of the PCK cholangiocytes in a dose-dependent fashion [17]. An inhibitor of epidermal growth factor receptor (EGFR) tyrosine kinase, gefitinib, also inhibits cell proliferation and induces apoptosis in the PCK cholangiocytes, leading to inhibition of cystic growth [18]. Importantly, these agents effective for inhibition of the PCK cyst growth in the 3-D culture system have been proven to ameliorate progressive cystic dilatation of the intrahepatic bile ducts of the PCK rat *in vivo* [15-18].

Pharmacologic activation of transient receptor potential vanilloid 4 (Trpv4), a calcium-entry channel, reverses the hyperproliferative phenotype and inhibits cystic growth of the PCK cholangiocytes by increasing intracellular calcium [19]. The pharmacologic activators of Trpv4 include 4α-phorbol 12,13-didecanoate, 5'6'-epoxyecosatrienoic acid, nifedipine and arachidonic acid. Overexpression and abnormal localization of the water channel aquaporin-1, the chloride channel cystic fibrosis transmembrane conductance regulator (CFTR) and the anion exchanger 2 in the PCK cholangiocytes may also be involved in increased fluid accumulation, and exposure of the PCK cholangiocytes in 3-D cultures to CFTR inhibitors [5-nitro-2-(3-phenylpropylamino)-benzoic acid and CFTRinh172] or Cl-/HCO3- exchange inhibitors (4,4'-diisothiocyanatostilbene-2,2'-disulfonic acid disodium salt hydrate and 4-acetamido-4'-isothiocyanato-2,2'-stilbenedisulfonic acid disodium salt hydrate) blocks secretin-stimulated fluid accumulation [20].

Elevated cAMP stimulates cholangiocyte proliferation via two downstream effectors, exchange proteins directly activated by cAMP (Epac1 and Epac2 isoforms) and protein kinase A, and intercellular calcium is involved in this process [21]. In fact, small interfering RNA (siRNA) against Epac1 and Epac2, as well as an inhibitor of mitogen-activated protein kinase kinases/extracellular signal-regulated kinase (MEK/ERK), U0126, significantly block the increased cyst growth of the PCK cholangiocytes in the 3-D culture system. PCK cholangiocyte hyperproliferation is also accompanied by the overexpression of Cdc25A protein and the downregulation of microRNA 15a [22]. MicroRNA 15a overexpression in the PCK cholangiocytes decreases Cdc25A levels and reduces cyst growth in the 3-D culture system.

Our recent data have shown that the signaling pathways involving phosphatidylinositol 3-kinase (PI3K)/AKT/mammalian target of rapamycin (mTOR) are activated in the PCK cholangiocytes, and an inhibitor of PI3K and mTOR complex1/2 (mTORC1/2), NVP-BEZ235, induces autophagy and reduces cyst growth of the PCK cholangiocytes in the 3-D culture system, while inhibitors of mTORC1, sirolimus and everolimus, do not reduce the cyst growth (Ren XS et al. unpublished data). The *in vivo* effects of NVP-BEZ235 have not been tested so far.

Pharmacotherapy for the PCK Rat *In Vivo*

Pharmacotherapies that can ameliorate liver and kidney lesions of the PCK rat *in vivo* have been investigated. The results available from the literatures are summarized in Table 2. There are relatively few therapeutic reagents that are effective for both liver and kidney lesions.

As expected from the results of the studies using the PCK cholangiocytes in the 3-D culture system, a reduction of the elevated cAMP by targeting somatostatin receptors (SSTRs) using octreotide and pasireotide inhibits cyst growth of the PCK liver as well as the kidney [15,16]. The natural SSTR ligand, somatostatin, is susceptible to proteolytic degradation and has s short half-life (~3 minutes), limiting its clinical utility. Octreotide binds with high affinity to SSTR2 and SSTR3, with moderate affinity to SSTR5, and has no affinity to SSTR1 and SSTR4 with a half-life of 2 hours. Octreotide improves cystogenesis as well as fibrosis of the liver and kidney of the PCK rat [15]. However, despite beneficial results, the effects of octreotide on liver and kidney volumes in preclinical and clinical trials are moderate in patients with polycystic liver disease (PLD) and ADPKD [35,36].

Pasireotide has a higher binding affinity to a broader range of SSTRs including SSTR1, SSTR2, SSTR3 and SSTR5, and a half-life of 12 hours. This long-acting somatostatin analog has been proven to be more effective than octreotide in reducing hepatorenal cystogenesis and fibrosis of the PCK rat, and a clinical trial to assess the effectiveness of pasireotide in hepatorenal cystogenesis in patients with PLD and ADPKD is now under way [16].

A Cdc25A inhibitor, vitamin K3, blocks cell-cycle progression and proliferation, reduces liver and kidney weights and cyst growth in the PCK rat [17]. Hyperproliferation of the PCK cholangiocytes is also associated with abnormalities in the EGFR axis involving the ErbB2 and B-Raf/MEK/ERK pathways, and c-Src is a critical mediator and co-factor in the activation and amplification of the EGFR axis. Inhibition of Src activity with SKI-606 is effective in ameliorating hepatorenal cystogenesis of the PCK rat without evidence of organ toxicity, and this occurs without reducing elevated cAMP [23]. It is of note that most of pharmacological reagents effective for improving hepatorenal cystogenesis of the PCK rat also improve the extent of liver and kidney fibrosis (Table 2), suggesting that hyperproliferation of cholangiocytes and renal tubular epithelial cells may be closely associated with tissue fibrosis.

Pioglitazone, an agonist of the peroxisome proliferator activator receptor gamma (PPARγ), inhibits cAMP-stimulated anion transport and the expression of Cl-channel, CFTR. *In vivo* administration of

Pharmaceutical	Potency	Biliary cystogenesis	Liver fibrosis	Renal cystogenesis	Renal fibrosis	Reference
Octreotide	Somatostatin analogue	+	+	+	+	[15]
Pasireotide	Somatostatin analogue	+	+	+	+	[16]
Vitamin K3	Cdc25A inhibitor	+	+	+	+	[17]
SKI-606	Srckinase inhibitor	+	+	+	nd	[23]
Pioglitazone	PPARγagonist	+	±	+	±	[24,25]
Gefitinib	EGFR tyrosine kinase inhibitor	+	+	-	-	[18]
OPC-31260	VPV2R antagonist	-	-	+	+	[26,27]
OPC-41061	VPV2R antagonist	-	-	+	+	[27]
GSK1016790A	Trpv4 activator	-	-	+	+	[19]
Lisinopril	ACE inhibitor	nd	nd	+	nd	[28]
HET-0016	20-HETE synthesis inhibitor	nd	nd	+	nd	[29]
Doxycycline	MMP inhibitor	nd	nd	+	nd	[30]
R-568	Type 2 calcimimetic	nd	nd	-	+	[31]
EKI-785	EGFR tyrosine kinase inhibitor	-	-	-	-	[32]
EKB-569	EGFR tyrosine kinase inhibitor	-	-	-	-	[32]
Sirolimus	mTORC1 inhibitor	-	-	-	-	[33]
Secretin	Adenylyl cyclase agonist	-	-	-	-	[34]

+, effective; -, not effective; nd, not determined.
2-HETE, 20-hydroxyeicosatetraenoic acid; ACE, angiotensin converting enzyme; EGFR, epidermal growth factor receptor; Cdc25A, cell division cycle 25A; MMP, matrix metalloproteinase; mTORC1, mammalian target of rapamycin complex 1; PPARγ, peroxisome proliferator activator receptor gamma; Trpv4, transient receptor potential vanilloid 4; VPV2R, vasopressin V2 receptor.

Table 2: Pharmacotherapy tested for ameliorating liver and kidney lesions of the PCK rat *in vivo*.

pioglitazone improves hepatorenal cystogenesis of the PCK rat, and the effects are accompanied by a decrease in the apical expression of CFTR in the bile duct epithelium [24]. As described above, the intrahepatic bile ducts of the PCK rat are constituted by two different phenotypes, bile ducts lined by cuboidal-shaped and flat-shaped cholangiocytes. It has been shown that the anti-proliferative effects of pioglitazone may be primarily manifested in the bile ducts lined by cuboidal-shaped cholangiocytes rather than those lined flat-shaped cholangiocytes [25].

An EGFR tyrosine kinase inhibitor, gefitinib, improves biliary cystogenesis and liver fibrosis of the PCK rat, while it has no beneficial effects on renal cyst development [18]. By contrast, other EGFR tyrosine kinase inhibitors, EKI-785 and EKB-569, have no significant effects on biliary cystogenesis as well as kidney lesions of the PCK rat [32].

The inhibition of renal cAMP production by treatment with vasopressin V2 receptor (VPV2R) antagonists, OPC-31260 and OPC41061, reduces plasma vasopressin, decreases cell proliferation and ameliorates renal cystogenesis with an associated reduction in B-Raf/MEK/ERK activity, leading to improved renal function in the PCK rat [26,27]. However, in consistent with the absence of VPV2R in the liver, it does not have a significant effect on biliary cystogenesis. Although Trpv4 activators listed in Table 1 inhibit cystic growth of the PCK cholangiocytes in the 3-D culture system, a specific Trpv4 activator, GSK 1016790A, has no significant effects of the biliary cystogenesis of the PCK rat *in vivo*, suggesting that at the low dose only kidney cystic cells are responsive to the agent [19].

In summary, pharmacological agents reported to be effective for biliary dysgenesis of the PCK rat until now are octreotide, pasireotide, vitamin K3, SKI-606, pioglitazone and genitinib. The other agents listed in Table 2 are not effective for the hepatobiliary lesions of the PCK rat, or the effects are not assessed in the literature.

Conclusion

Studies have revealed that biliary dysgenesis is associated with cholangiocyte hyperproliferation, cell-matrix interactions and accelerated fluid transport. The levels of cAMP and intracellular calcium are closely associated with the pathogenesis, and the several key signaling pathways including the activation of the B-Raf/MEK/ERK signaling pathway have been identified. Although the cystogenesis of the liver and kidney appear to share similar pathogenesis, several differences exist. It seems likely that more effective therapeutic approaches will require combination therapies affecting multiple cystogenesis pathways.

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