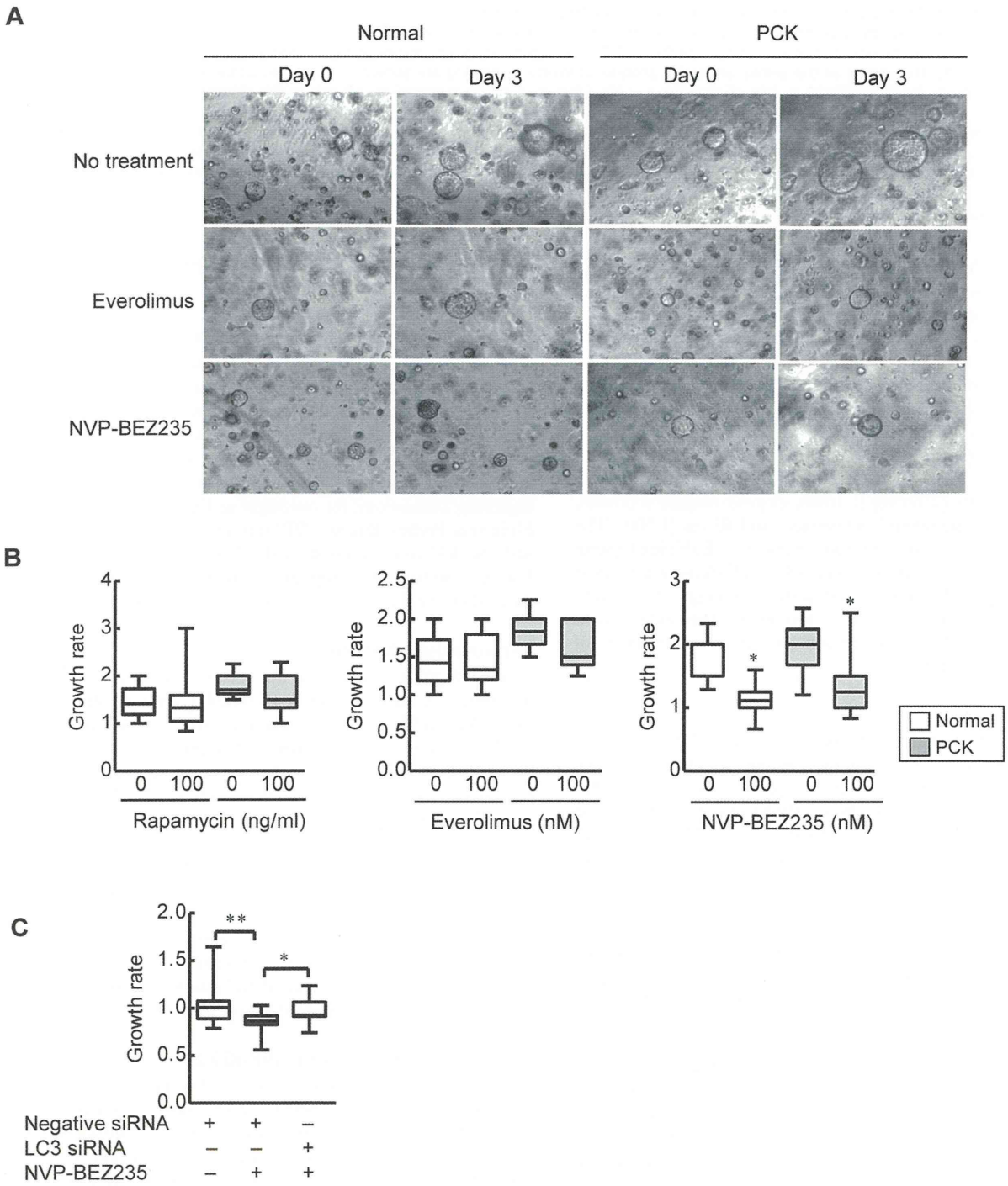

Figure 5. Inhibition of biliary cyst formation by mTOR inhibitors in vitro. The effects of mTOR inhibitors on biliary cyst formation were examined for normal and PCK cholangiocytes using three-dimensional cell culture system as described in the Materials and Methods. PCK cholangiocytes grew more rapidly in a spheroidal form in the Matrigel compared to normal cholangiocytes under normal condition (A). NVP-BEZ235 significantly inhibited cystic growth of both normal and PCK cholangiocytes, while rapamycin and everolimus did not have significant inhibitory effects on biliary cyst formation of the cholangiocytes (B). Knockdown of LC3 with siRNA of PCK cholangiocytes that were treated with NVP-BEZ235 significantly increased the biliary cyst formation (C). *, $p<0.01$ (vs. untreated control) (B): *, $p<0.01$; **, $p<0.05$ (C).
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fibrosis was assessed using picrosirius red staining. Stained sections were visualized under a light microscope, and the digital images were acquired and reproduced on a computer. Image analysis was performed in software using Adobe Photoshop (Adobe Systems Incorporated, San Jose, CA). A color threshold was applied at a level that separated cysts from non-cystic tissue or the picrosirius red stained material from the background to calculate the volume of the cysts or fibrosis. The areas of interest were expressed as a percentage of the total tissue.

Table 1. Treatment of normal and PCK rats with NVP-BEZ235.

	Normal no treatment	Normal NVP-BEZ235	PCK no treatment	PCK NVP-BEZ235
Number of rats	6	5	5	6
Body weight (g)	340±5	275±7*	337±11	291±5**
Liver/body weight (%)	4.4±0.1	4.3±0.3	6.1±0.5	5.6±0.1
Kidney/body weight (%)	0.86±0.02	0.84±0.03	1.19±0.10	1.03±0.00
Aspertate aminotransferase (IU/L)	85±3	94±5	91±14	88±6
Alkaline phosphatase (U/L)	1413±111	1040±169	1174±114	994±48
Total protein (g/dL)	5.9±0.1	5.8±0.0**	5.5±0.3	5.7±0.1
Albumin (g/dL)	4.5±0.1	4.4±0.1	3.2±0.1	3.9±1.0*
Blood urea nitrogen (mg/dL)	17±1	16±2	20±2	17±0
Cretinine (mg/dL)	0.30±0.00	0.32±0.02	0.40±0.03	0.32±0.02

*, $p<0.01$;
**, $p<0.05$ (vs. untreated control).
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Evaluation of apoptosis was performed using liver sections stained using the TUNEL method. More than 500 biliary epithelial cells were surveyed in the sections, and the percentage of biliary epithelial cells positive for TUNEL was expressed as the TUNEL-labeling index.

To evaluate the cell proliferative activity, Ki-67 protein-positive signals were similarly counted for biliary epithelial cells of the Ki-67-immunostained liver sections, and the percentage of biliary epithelial cells positive for Ki-67 protein were expressed as the Ki-67-labeling index.

Statistics

The mean ± SD was calculated for all parameters. Statistical differences were determined using the T-test and analysis of variance. A p value <0.05 was accepted as the level of statistical significance.

Results

Overexpression of p-mTOR, p-Akt, p-S6 and PI3K in PCK Cholangiocytes

The immunohistochemical expression of p-mTOR (Ser2448) and p-Akt (Ser473) was examined using liver sections of 10-month-old rats. In the normal rat, the expression of p-mTOR (Ser2448) in bile duct epithelium was faint or negligible, and that of p-Akt (Ser473) was weak (Figure 1A, arrows). In the PCK liver, the expression of both p-mTOR (Ser2448) and p-Akt (Ser473) in bile duct epithelium was markedly increased (Figure 1A).

Western blot analysis showed that the expression p-mTOR (Ser2448), p-mTOR (Ser2481), p-Akt (Ser473), p-S6, PI3K p100α, and PI3K p85 was increased in cultured PCK cholangiocytes compared to normal cholangiocytes (Figure 1B and 1C), where the expression p-mTOR (Ser2448) was indicative of the activation of mTORC1, and p-mTOR (Ser2481) and p-Akt (Ser473) were indicative of the activation of mTORC2. The expression of p-ERK1/2 was not significantly different between normal and PCK cholangiocytes (data not shown).

In vitro Effects of PI3K and mTOR Inhibitors on Cholangiocytes

Normal and PCK cholangiocytes were treated with rapamycin (mTORC1 inhibitor), everolimus (mTORC1 inhibitor), NVP-BEZ235 (PI3K and mTORC1/2 inhibitor) and LY294002 (PI3K inhibitor), and the cell proliferative activity was determined using the WST-1 assay. Each inhibitor significantly inhibited the cell proliferative activity of normal (Figure 2A) and PCK (Figure 2B) cholangiocytes in a dose-dependent fashion. Above all, NVP-BEZ235 had most prominent inhibitory effects on the cell proliferative activity of both cholangiocytes, and at the concentration of 500 nM, it reduced the cell proliferative activity of both cholangiocytes to less than 30% compared to that of untreated groups at 120 hours (Figure 2A and 2B). Higher concentrations of rapamycin (10 μg/ml) and everolimus (10 μM) showed almost same inhibitory effects on the cell proliferative activity as those seen in 100 ng/ml rapamycin and 100 nM everolimus, respectively (data not shown).

To address the role of mTORC2 in cholangiocyte proliferation, the experiment of depletion of Rictor using siRNA was performed. Western blot analysis confirmed the gene silencing for Rictor was efficiently conducted (Figure 2C, upper panel). Although the Rictor siRNA alone did not affect the cell proliferative activity of PCK cholangiocytes, the cell proliferative activity was further reduced when the siRNA was used in combination with rapamycin and LY294002 (Figure 2C). Thus, mTORC2 was partially involved in the increased cell proliferation of PCK cholangiocytes.

Western blot analysis showed that the expression of p-Akt (Se473) and p-S6 was reduced following the treatment with rapamycin, everolimus and NVP-BEZ235 in a dose-dependent fashion in PCK cholangiocytes, and NVP-BEZ235 further reduced the expression of p-4E-BP1 (Figure 2C and 2D). Higher concentration (500 nM) of NVP-BEZ235 almost completely diminished the expression of p-Akt (Ser473).

Effects of mTOR Inhibitors on Cholangiocyte Apoptosis in vitro

Apoptosis was determined following the treatment of PCK cholangiocytes with rapamycin, everolimus and NVP-BEZ235. Flow cytometric analysis showed that rapamycin and everolimus significantly induced apoptosis in PCK cholangiocytes (Figure 3A). By contrast, apoptosis was significantly inhibited by the treatment with NVP-BEZ235 (Figure 3A).

In relation to the inhibition of apoptosis, NVP-BEZ235 significantly reduced the expression of cleaved caspase 3 in PCK cholangiocytes, while it tended to be unchanged or rather increased following the treatment with everolimus (Figure 3B

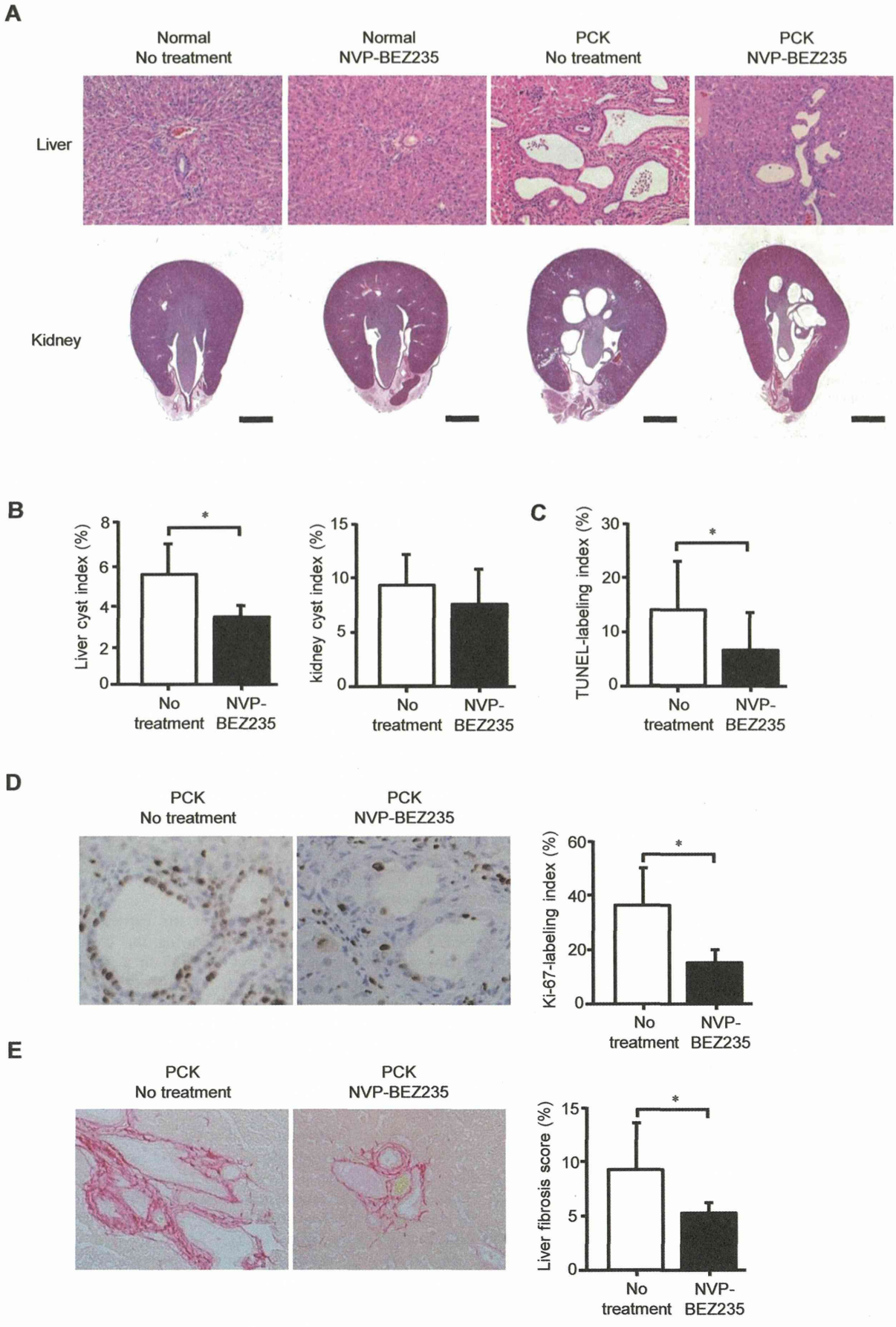
A
Normal
No treatment
Normal
NVP-BEZ235
PCK
No treatment
PCK
NVP-BEZ235
Liver
Kidney
B
Liver cyst index (%)
kidney cyst index (%)
No treatment
NVP-BEZ235
C
TUNEL-labeling index (%)
D
Ki-67-labeling index (%)
E
Liver fibrosis score (%)

Figure 6. Effects of in vivo administration of NVP-BEZ235 on liver and renal diseases of the PCK rat. Normal and PCK rats were treated with NVP-BEZ235 or vehicle alone daily between 4 and 8 weeks of age. Liver and kidney sections stained with hematoxylin-eosin showed that NVP-BEZ235 improved dilatation of intrahepatic bile ducts of the PCK rat, but no beneficial effects were observed on kidney lesions (A). For the PCK rat, liver and kidney cyst index (B), TUNEL-labeling index of the biliary epithelial cells (C), Ki-67-labeling index of the biliary epithelial cells (D), and liver fibrosis score (E) were determined as described in the Materials and Methods. Treatment with NVP-BEZ235 significantly reduced liver cyst index, TUNEL-labeling index, Ki-67-labeling index and liver fibrosis score of the PCK rat, whereas kidney cyst index was unaffected. Original magnifications: x200 (A, upper panel; E); x400 (D). Bars, 2 mm (A, lower panel). *, $p<0.01$.
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and 3C). Silencing of caspase 3 using siRNA in PCK cholangiocytes reduced the percentage of apoptotic cells from 10.04% to 7.72% under normal condition, and from 45.16% to 25.28% under H_2O_2-treated condition, confirming that caspase 3 was involved in the induction of apoptosis in PCK cholangiocytes (Figure 3D). In consistent with the inhibition of apoptosis by NVP-BEZ235, the analysis with quantitative real-time PCR showed that treatment with NVP-BEZ235 significantly increased the expression of bcl-2 mRNA in PCK cholangiocytes (Figure 3E).

Effects of mTOR Inhibitors on Cholangiocyte Autophagy in vitro

The occurrence of autophagy in cholangiocytes was detected by the conversion of LC3 I to LC3 II. Western blot analysis showed that NVP-BEZ235 induced a dose-dependent increase in the autophagy-specific LC3 II form, while rapamycin and everolimus did not significantly induce autophagy in PCK cholangiocytes (Figure 4A and 4B). Immunocytochemistry of LC3 confirmed the induction of autophagy by NVP-BEZ235 (Figure 4C).

To further determine the involvement of autophagy in the inhibition of the cell proliferative activity by NVP-BEZ235, silencing of LC3 was performed using LC3 siRNA in PCK cholangiocytes, and the cell proliferative activity was compared for the cells with and without NVP-BEZ235 treatment. The gene silencing was monitored using immunocytochemistry and Western blotting for PCK cholangiocytes maintained with serum-free medium, and the analysis showed that LC3 siRNA reduced LC3 II formation in the cholangiocytes, although their expression was not completely diminished (Figure 4D and 4E). As expected, when PCK cholangiocytes were treated with NVP-BEZ235, the cell proliferative activity of the LC3 siRNA-treated cells was significantly high than those without LC3 siRNA treatment (Figure 4F). When PCK cholangiocytes were treated with 3MA, a specific inhibitor of autophagy, together with NVP-BEZ235, the cell proliferative activity was significantly increased compared to those with NVP-BEZ235 alone (Figure 4G), confirming that autophagy was involved in the growth inhibition by NVP-BEZ235 in PCK cholangiocytes.

Inhibition of Biliary Cyst Formation by mTOR Inhibitors in vitro

The effects of mTOR inhibitors on biliary cyst formation were examined using three-dimensional cell culture system. PCK cholangiocytes grew more rapidly in a spheroidal form in the Matrigel compared to normal cholangiocytes under normal condition (Figure 5A). NVP-BEZ235 significantly inhibited cystic growth of both normal and PCK cholangiocytes (Figure 5A and 5B). Knockdown of LC3 with siRNA of PCK cholangiocytes that were treated with NVP-BEZ235 significantly increased the biliary cyst formation (Figure 5C), showing further evidence of the involvement of autophagy in these processes. By contrast, rapamycin and everolimus did not have significant inhibitory effects on biliary cyst formation of both normal and PCK cholangiocytes (Figure 5A and 5B).

In vivo Administration of NVP-BEZ235

Finally, the effects of in vivo administration of NVP-BEZ235 on the progression of liver and renal diseases in the PCK rat were examined. Hematological analysis showed that liver and renal functions were preserved following the treatment, but retardation in body weight increase was noted in both normal and PCK rats by the treatment (Table 1). As expected, the treatment reduced the extent of dilatation of intrahepatic bile ducts of the PCK rat, whereas no apparent histological changes were observed in the liver of normal rats (Figure 6A). On the contrary, no beneficial effects were observed on renal cyst development because of the treatment as evaluated using routine histological sections (Figure 6A). Histomorphometric analysis using whole liver and kidney tissues confirmed that the treatment significantly reduced liver cyst index, but not kidney cyst index (Figure 6B).

In the liver of the PCK rat, apoptosis of biliary epithelial cells was significantly inhibited by the treatment (Figure 6C), while the cell proliferative activity of the cells was significantly reduced (Figure 6D), which were in consistent with the results of in vitro experiments. In addition, the treatment significantly improved the extent of liver fibrosis of the PCK rat (Figure 6E).

Immunohistochemical analysis confirmed that the expression of p-Akt (Ser473), p-mTOR (Ser2448) and p-S6 was reduced in bile duct epithelium of the PCK rat treated with NVP-BEZ235 (Figure 7A). The treatment increased immunohistochemical expression of LC3 in bile duct epithelium of the PCK rat (Figure 7B), suggesting the occurrence of autophagy.

In the kidney, the expression of p-Akt (Ser473), p-mTOR (Ser2448) and p-S6 was increased in collecting tubule-derived cyst epithelium of the PCK rat without NVP-BEZ235 treatment compared to those in the renal tubules of normal rat (Figure 7C). Although the treatment had no significant effect on kidney cyst index, the immunohistochemical expression of these molecules appeared to be reduced in the kidney of the PCK rat following the treatment (Figure 7C).

Discussion

This study demonstrated that the PI3K/Akt/mTOR pathway was activated in PCK cholangiocytes. The treatment with NVP-BEZ235 effectively inhibited cholangiocyte cystic proliferation by enhancing autophagy and inhibiting apoptosis in vitro, and the inhibitory effects were also observed in the PCK rat treated with NVP-BEZ235 in vivo. Rapamycin and everolimus induced apoptosis of the cells, but they could not inhibit cystic growth of the cells in the three-dimensional cell culture system.

A recent study showed that in vivo administration of rapamycin failed to inhibit cystic dilatation of the bile ducts of the PCK rat, and the authors considered that this might be due to intrinsic or acquitted rapamycin resistance [14]. In clinical trials where rapamycin and everolimus did not show beneficial effects in patients with ADPKD, low tissue concentrations of the drugs at clinically tolerable doses have been considered as one of the causes [12,13]. Our data suggest that the inhibition of mTORC1 alone may be insufficient for the inhibition of cell proliferation of PCK cholangiocytes, because other complementary signaling pathways

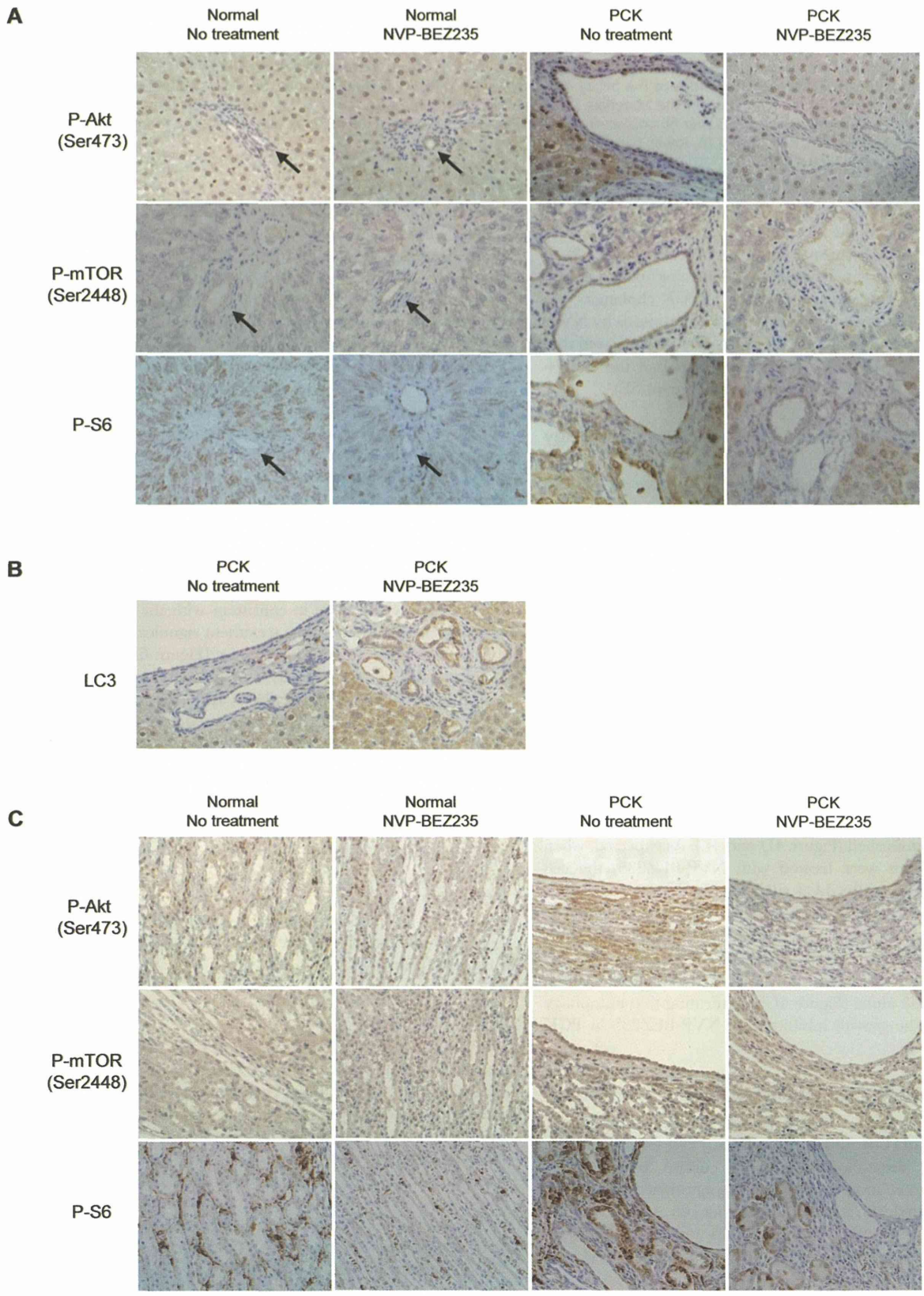

Figure 7. Immunohistochemical analysis of the the liver and kidney of the PCK rat treated with NVP-BEZ235. Treatment with NVP-BEZ235 reduced the expression of p-Akt (Ser473), p-mTOR (Ser2448) and p-S6 in bile duct epithelium of the PCK rat (A). The treatment increased immunohistochemical expression of LC3 in bile duct epithelium of the PCK rat (B). In the kidney, the expression of p-Akt (Ser473), p-mTOR (Ser2448) and p-S6 was increased in collecting tubule-derived cyst epithelium of the PCK rat without NVP-BEZ235 treatment compared to those in the renal tubules of normal rat, and their expression appeared to be reduced in the kidney of the PCK rat following the treatment (C). Arrows indicate interlobular bile ducts of the normal liver. Original magnifications; x400 (A–C).
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were activated in PCK cholangiocytes. More prominent inhibitory effects of NVP-BEZ235 rather than rapamycin and everolimus on the cell proliferation of PCK cholangiocytes support that the inhibition of other signaling pathways such as PI3K may be important for inhibiting the cell proliferative activity.

Drug-induced growth inhibition is often associated with increased apoptosis in PKD cells, and apoptosis has been implicated in strategies for therapeutic intervention [21]. However, the studies of the effects of rapamycin on apoptosis in PKD are conflicting [26], and the present study showed that rapamycin and everolimus induced apoptosis in PCK cholangiocytes. By contrast, NVP-BEZ235 inhibited apoptosis by reducing the expression of cleaved caspase 3 and by inducing bcl-2 expression. In several cancer cell lines, it has been shown that NVP-BEZ235 induced apoptosis and cell cycle arrest accompanied by increased caspase 3 activity, and bcl-2 abrogated the effects of NVP-BEZ235 [27–29]. Regarding the effects of NVP-BEZ235 on apoptosis in PKD cells, limited data are available, and further detailed studies are required to clarify the mechanism of the inhibition of apoptosis in PCK cholangiocytes by NVP-BEZ235.

In the kidney, a recent study showed an increase in LC3 II and beclin 1 expression in the late stages of PKD in rodent models of ADPKD [22]. In consistent with the facts that autophagy has been described as hypoxia-inducible factor-1α-dependent adaptive response, HIF-1α was also highly expressed in the late stages of PKD in relation to the occurrence of autophagy [22]. In their studies, however, the hypoxia-inducible factor-1α inhibitor had no significant effects on kidney lesions in vivo. To date, published data on the involvement of autophagy is unavailable for the disease pathogenesis of kidney and liver lesions of ARPKD as well as Caroli's disease and their rodent models.

The mTOC1 is a negative regulator of autophagy, directly by phosphorylating ULK1 and preventing ULK1-Atg13-FIP200 complex formation, and indirectly by phosphorylating S6 and 4E-BP1 [30]. Recently, reports on the involvement of mTORC2 in autophagy have started to emerge, and mTORC2 regulates autophagy by affecting forkhead box O and regulating some key autophagy genes such as LC3 and beclin 1 [31]. In these conditions, rapamycin is unable to induce autophagy. The regulation of autophagy process by mTORC1/2 suggests that the inhibition of mTOR kinase activity, by having an impact on both mTOR complexes, may have a greater effect on autophagy, compared to rapamycin alone. Indeed, NVP-BEZ235, not rapamycin and everolimus, significantly induced autophagy in PCK cholangiocytes.

In this study, rapamycin, everolimus and NVP-BEZ235 blocked the phosphorylation of Akt (Ser473) and the downstream effectors of mTORC1/2, S6 and 4E-BP1, in PCK cholangiocytes. The inhibition of the phosphorylation of Akt (Ser473) by the mTORC1 inhibitors was consistent with the reported findings showing that higher concentration of rapamycin might target mTORC2 [32]. It was of note that higher concentration (500 nM) of NVP-BEZ235 markedly reduced the expression of p-Akt (Ser473) and p-4E-BP1 in PCK cholangiocytes. Since recent studies have shown that 4E-BP1 is a potential new target molecule for anti-cancer therapy [33], the marked inhibitory effects of NVP-BEZ235 on the phosphorylation of 4E-BP1 as well as Akt (Ser473) further support its applicability as a therapeutic agent.

As expected, the treatment of the PCK rats with NVP-BEZ235 significantly inhibited cystic dilatation of the intrahepatic bile ducts in vivo, whereas renal cyst development was not affected by the treatment. Similarly, an epidermal growth factor tyrosine kinase inhibitor (gefitinib) significantly has been shown to reduce cystic bile duct dilatation of the PCK rat in vivo, but kidney lesions were unaffected or rather worsened according to our previous study [34]. These results suggest that the mechanism of cyst progression of the PCK rat may be different between the liver and kidney. In addition, in vivo administration of NVP-BEZ235 caused retardation in body weight increase in the rats, indicating the limitation of clinical application of this agent for the patients with ARPKD, at least at the dosage and the treatment period used in this study.

In conclusion, this study showed the involvement of aberrant activation of the PI3K/mTOR pathway in the dilatation of intrahepatic bile ducts of the PCK rat. Although the PI3K/Akt/mTOR signaling pathway represents a complex network, inhibition of the pathway using specific inhibitors attenuates cystic proliferation of PCK cholangiocytes via the mechanism involving apoptosis and/or autophagy of the cells, which is preferably further addressed for therapeutic purpose.

Author Contributions

Conceived and designed the experiments: JYS YN. Performed the experiments: XSR YS. Analyzed the data: KH MS. Contributed reagents/materials/analysis tools: YS SF. Wrote the paper: XSR.

References

1. Nakanuma Y, Harada K, Sato Y, Ikeda H (2010) Recent progress in the etiopathogenesis of pediatric biliary disease, particularly Caroli's disease with congenital hepatic fibrosis and biliary atresia. Histol Histopathol 25: 223–235.
2. Harris PC, Torres VE (2009) Polycystic kidney disease. Annu Rev Med 60: 321–337.
3. Sanzen T, Harada K, Yasoshima M, Kawamura Y, Ishibashi M, et al. (2001) Polycystic kidney rat is a novel animal model of Caroli's disease associated with congenital hepatic fibrosis. Am J Pathol. 158: 1605–1612.
4. Sato Y, Ren XS, Nakanuma Y (2012) Caroli's disease: current knowledge of its biliary pathogenesis obtained from an orthologous rat model. Int J Hepatol.
5. Zhou H, Huang S (2010) The complexes of mammalian target of rapamycin. Curr Protein Pept Sci. 11: 409–424.
6. Copp J, Manning G, Hunter T (2009) TORC-specific phosphorylation of mammalian target of rapamycin (mTOR): phospho-Ser2481 is a marker for intact mTOR signaling complex 2. Cancer Res. 69: 1821–1827.
7. Spirli C, Okolicsanyi S, Fiorotto R, Fabris L, Cadamuro M, et al. (2010) Mammalian target of rapamycin regulates vascular endothelial growth factor-dependent liver cyst growth in polycystin-2-defective mice. Hepatology. 51: 1778–1788.
8. Shillingford JM, Piontek KB, Germino GG, Weimbs T (2010) Rapamycin ameliorates PKD resulting from conditional inactivation of Pkd1. J Am Soc Nephrol. 21: 489–497.
9. Zafar I, Ravichandran K, Belibi FA, Doctor RB, Edelstein CL (2010) Sirolimus attenuates disease progression in an orthologous mouse model of human autosomal dominant polycystic kidney disease. Kidney Int. 78: 754–761.
10. Walz G (2006) Therapeutic approaches in autosomal dominant polycystic kidney disease (ADPKD): is there light at the end of the tunnel? Nephrol Dial Transplant 21: 1752–1757.
11. Shillingford JM, Murcia NS, Larson CH, Low SH, Hedgepeth R, et al. (2006) The mTOR pathway is regulated by polycystin-1, and its inhibition reverses renal cystogenesis in polycystic kidney disease. Proc Natl Acad Sci U S A 103: 5466–5471.
12. Serra AL, Poster D, Kistler AD, Krauer F, Raina S, et al. (2010) Sirolimus and kidney growth in autosomal dominant polycystic kidney disease. N Engl J Med. 363: 820–829.
13. Walz G, Budde K, Mannaa M, Nürnberger J, Wanner C, et al. (2010) Everolimus in patients with autosomal dominant polycystic kidney disease. N Engl J Med. 363: 830–840.
14. Renken C, Fischer DC, Kundt G, Gretz N, Haffner D (2011) Inhibition of mTOR with sirolimus does not attenuate progression of liver and kidney disease in PCK rats. Nephrol Dial Transplant. 26: 92–100.
15. Fischer DC, Jacoby U, Pape L, Ward CJ, Kuwertz-Broeking E, et al. (2009) Activation of the AKT/mTOR pathway in autosomal recessive polycystic kidney disease (ARPKD). Nephrol Dial Transplant 24: 1819–1827.
16. Chiarini F, Grimaldi C, Ricci F, Tazzari PL, Evangelisti C, et al. (2010) Activity of the novel dual phosphatidylinositol 3-kinase/mammalian target of rapamycin

inhibitor NVP-BEZ235 against T-cell acute lymphoblastic leukemia. Cancer Res 70: 8097–8107.
17. Liu TJ, Koul D, LaFortune T, Tiao N, Shen RJ, et al. (2009) NVP-BEZ235, a novel dual phosphatidylinositol 3-kinase/mammalian target of rapamycin inhibitor, elicits multifaceted antitumor activities in human gliomas. Mol Cancer Ther. 8: 2204–2210.
18. Zhou XJ, Kukes G (1998) Pathogenesis of autosomal dominant polycystic kidney disease: role of apoptosis. Diagn Mol Pathol 7: 65–68.
19. Lanoix J, D'Agati V, Szabolcs M, Trudel M (1996) Dysregulation of cellular proliferation and apoptosis mediates human autosomal dominant polycystic kidney disease (ADPKD). Oncogene 13: 1153–1160.
20. Woo D (1995) Apoptosis and loss of renal tissue in polycystic kidney diseases. N Engl J Med 333: 18–25.
21. Hu B, He X, Li A, Qiu Q, Li C, et al. (2011) Cystogenesis in ARPKD results from increased apoptosis in collecting duct epithelial cells of Pkhd1 mutant kidneys. Exp Cell Res 317: 173–187.
22. Belibi F, Zafar I, Ravichandran K, Segvic AB, Jani A, et al. (2011) Hypoxia-inducible factor-1α (HIF-1α) and autophagy in polycystic kidney disease (PKD). Am J Physiol Renal Physiol. 300:F1235–1243.
23. Sato Y, Harada K, Kizawa K, Sanzen T, Furubo S, et al. (2005) Activation of the MEK5/ERK5 cascade is responsible for biliary dysgenesis in a rat model of Caroli's disease. Am J Pathol 166: 49–60.
24. Ren XS, Sato Y, Harada K, Sasaki M, Yoneda N, et al. (2011) Biliary infection may exacerbate biliary cystogenesis through the induction of VEGF in cholangiocytes of the polycystic kidney (PCK) rat. Am J Pathol. 179: 2845–2854.
25. Evert M, Calvisi DF, Evert K, De Murtas V, Gasparetti G, et al. (2012) V-AKT murine thymoma viral oncogene homolog/mammalian target of rapamycin activation induces a module of metabolic changes contributing to growth in insulin-induced hepatocarcinogenesis. Hepatology. 55: 1473–1484.
26. Edelstein CL (2008) Mammalian target of rapamycin and caspase inhibitors in polycystic kidney disease. Clin J Am Soc Nephrol. 3: 1219–1226.
27. Masuda M, Shimomura M, Kobayashi K, Kojima S, Nakatsura T (2011) Growth inhibition by NVP-BEZ235, a dual PI3K/mTOR inhibitor, in hepatocellular carcinoma cell lines. Oncol Rep. 26: 1273–1279.
28. Santiskulvong C, Konecny GE, Fekete M, Chen KY, Karam A, et al. (2011) Dual targeting of phosphoinositide 3-kinase and mammalian target of rapamycin using NVP-BEZ235 as a novel therapeutic approach in human ovarian carcinoma. Clin Cancer Res. 17: 2373–2384.
29. Muranen T, Selfors LM, Worster DT, Iwanicki MP, Song L, et al. (2012) Inhibition of PI3K/mTOR leads to adaptive resistance in matrix-attached cancer cells. Cancer Cell. 21: 227–239.
30. Wang X, Proud CG (2011) mTORC1 signaling: what we still don't know. J Mol Cell Biol. 3: 206–220.
31. Sini P, James D, Chresta C, Guichard S (2010) Simultaneous inhibition of mTORC1 and mTORC2 by mTOR kinase inhibitor AZD8055 induces autophagy and cell death in cancer cells. Autophagy. 6: 553–554.
32. Foster DA, Toschi A (2009) Targeting mTOR with rapamycin: one dose does not fit all. Cell Cycle. 8: 1026–1029.
33. Nawroth R, Stellwagen F, Schulz WA, Stoehr R, Hartmann A, et al. (2011) S6K1 and 4E-BP1 are independent regulated and control cellular growth in bladder cancer. PLoS One. 6:e27509.
34. Sato Y, Harada K, Furubo S, Kizawa K, Sanzen T, et al. (2006) Inhibition of intrahepatic bile duct dilation of the polycystic kidney rat with a novel tyrosine kinase inhibitor gefitinib. Am J Pathol. 169: 1238–1250.

特集1　臨床・画像・病理トライアングル（第33回日本画像医学会）

◆肝細胞癌と鑑別を要する疾患

病理学的に肝細胞癌と鑑別を要する疾患

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はじめに

病理学的に肝細胞癌やDysplastic noduleと鑑別を要する肝細胞性の結節性病変として、①Hepatocellular adenoma（HCA）、②Focal nodular hyperplasia（FNH）およびFNH-like nodule、③FNH-like nodule（hypervascular hyperplastic nodule）in heavy drinker、④Large regenerative nodule（LRN）、Macroregenerative nodule（MRN）、⑤Partial nodular transformation（PNT）、⑥Nodular regenerative hyperplasia（NRH）、⑦Combined hepatocellular-cholangiocarcinoma with stem-cell featuresがある。また、非肝細胞性の重要な鑑別疾患として血管筋脂肪腫が重要である。

肝細胞腺腫（HCA）

HCAは経口避妊薬の服用歴のある若年女性に好発（85％）するが、本邦では蛋白同化ホルモン服用、Ⅰ型糖原病などの代謝障害、肥満、アルコール多飲歴を有する症例が多い。通常、正常肝から発生する境界明瞭な単発性の良性腫瘍で、正常肝細胞に類似した異型の乏しい細胞の増生よりなり、腫瘍結節内に門脈域、胆管を欠く。また、多数の異常筋性血管も見られ多血性結節として描出される。新WHO分類（2010）で、HCAは遺伝子型をもとに①hepatocyte nuclear factor（HNF）1A遺伝子変異によるHNF1α不活性型、②β-catenin遺伝子変異による恒常的なβ-catenin活性化型、③gp130遺伝子等変異によるIL-6/gp130/STAT系の恒常的な活性化を示す炎症性型、④分類不能型に亜分類され、それぞれ変異遺伝子を反映した免疫組織化学的鑑別が容易となった[1]。同時に各亜型の臨床病理学的特徴も明らかとなりつつあるが、慢性肝障害併存例からの発症例、早期または高分化型肝細胞癌との異同[2]、複数の遺伝子変異パターンを示す症例の存在など従来のHCAの概念および診断基準とのすり合わせが今後必要である。

限局性結節性過形成（FNH）

FNHは非硬変肝（通常 正常肝）に発生し、境界明瞭な数mm～15cm以上の結節を呈する。

病理学的に、異型のない肝細胞の過形成からなり、結節中心部に中心性瘢痕と放射状に伸びる線維性隔壁が特徴であり（**図1**）、隔壁内、実質内に異常筋性血管や隔壁に近接して細胆管増生、リンパ球浸潤、慢性胆汁うっ滞（肝細胞の淡明腫大、銅顆粒の沈着）を認める。glutamine synthetaseの免疫染色にて地図様のパターンを示すのが特徴である（**図1**）[1]。一方、中心性瘢痕を伴わないFNH症例（FNH-like nodule）もしばしば経験され、近年、アルコール多飲者にみられる過形成結節としても注目されている。

混合型肝癌

新WHO分類（2010）では、典型的な肝細胞癌と胆管癌の成分の混在が確認できる混合型肝癌“classical type”以外に、ステム細胞的な特徴を併

図1A 図1B

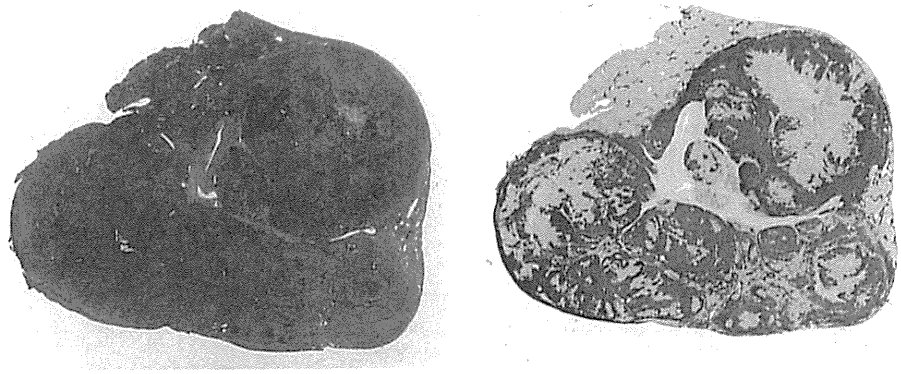

図1　Focal nodular hyperplasia (FNH) 症例
中心性瘢痕 (A：矢印) を有する典型的なFNHで、Glutamine synthetase (GS) の免疫染色ではMap-like patternを呈する (B)。
IMAGE PREVIEW P.406参照

図2A 図2B

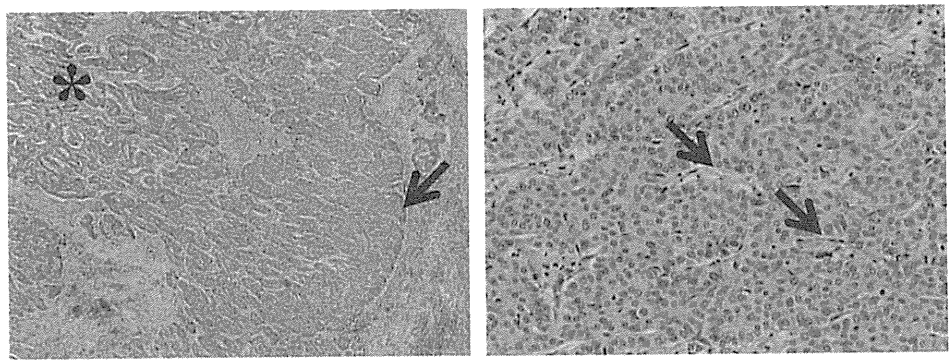

図2　Combined hepatocellular-cholangiocarcinoma with stem-cell features (いわゆる細胆管癌)
A：腺管構造を示すいわゆる細胆管癌 (*) 成分と腫瘍辺縁には境界明瞭な充実性の腫瘍成分を認める (矢印)。
B：腫瘍辺縁の充実性成分では、類洞様のスリット状血管 (矢印) も散見され、肝細胞癌に類似する組織像を呈する。
IMAGE PREVIEW P.406参照

せもつ混合型肝癌として"Combined hepatocellular-cholangiocarcinoma with stem-cell features"が新たなカテゴリとして設けられ、①typical type、②intermediate-cell type、③cholangiolocellular typeの3つのサブタイプに細分類された[3]。このうちcholangiolocellular type (いわゆる細胆管癌に相当) は、画像上、多血性結節を呈する症例や、組織学的に類洞様構造を伴う肝細胞癌に酷似した形態を示す症例がある (図2)。しかし、肝細胞マーカ (HepPAR1) 陰性、胆管マーカ (CK19) 陽性であり、診断には免疫染色による確認が重要である。

血管筋脂肪腫

血管筋脂肪腫は、血管、平滑筋細胞、脂肪組織が混在した間葉系腫瘍であり、病理学的に被膜はないが、境界明瞭な腫瘍で各構成成分の多寡により多彩な割面を呈する。しかし、平滑筋細胞のみ

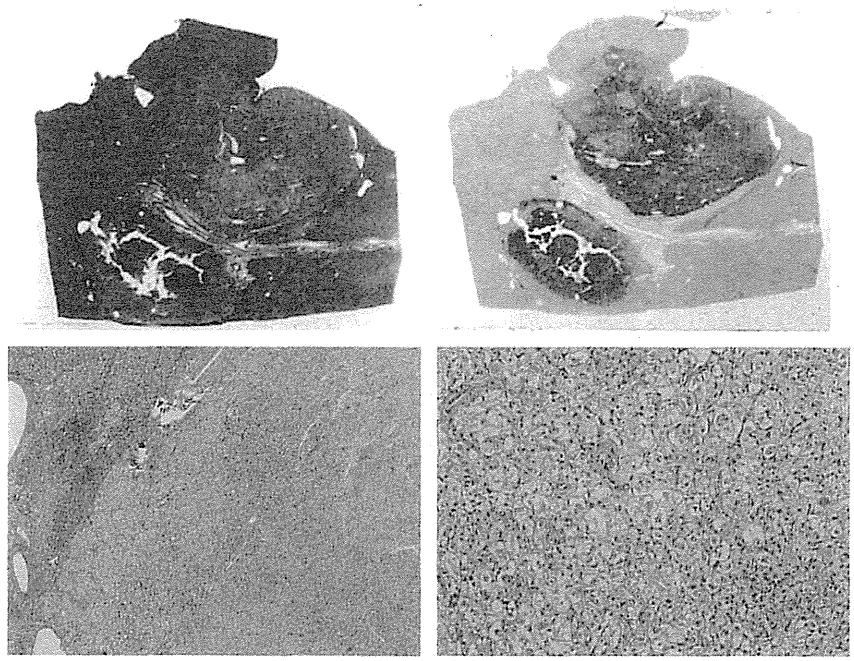

図3　血管筋脂肪腫　　図3A | 図3B / 図3C | 図3D

A：境界明瞭な充実性の腫瘍で、脂肪細胞成分からなる明るい領域は認めない。
B：メラニンマーカであるHMB45がびまん性に陽性。
C：境界明瞭な充実性の腫瘍で、脂肪成分は認めない。
D：強拡大では、肝細胞類似の胞体の明るい細胞からなる。

IMAGE PREVIEW P.407参照

からなるMonotypic epithelioid angiomyolipoma (Perivascular epithelioid cell tumor, PEComa)[4]は、好酸性胞体を有する類上皮様細胞が索状に増生し、肝細胞癌に酷似するが、メラノサイトマーカであるHMB45, Melan Aの免疫染色が診断の決め手となる（図3）。

さいごに

肝細胞癌との鑑別を要する肝病変について病理学的視点から概説した。病理学的診断に際し、免疫染色は強力なツールであることは間違いないが、染色結果を過信することなく診断する必要がある。また、従来の診断基準との矛盾症例や非定型例が存在し、さらに腫瘍の一部しか見ることができない腫瘍針生検では、診断困難な症例も多い。

参考文献

1) Bioulac-Sage P et al: Focal nodular hyperplasia and hepatocellular adenoma. In: Bosman FT, Carneiro F, Hruban RH, Theise ND, eds. WHO classification of tumors of the digestive system; World Health Organization of Tumors. 4th ed. Lyon: IARC 198-204, 2010

2) Evason KJ et al: Atypical hepatocellular adenoma-like neoplasms with beta-catenin activation show cytogenetic alterations similar to well-differentiated hepatocellular carcinomas. Hum Pathol 44(5): 750-758, 2013

3) Theise ND et al: Combined hepatocellular-cholangiocarcinoma. In: Bosman FT, Carneiro F, Hruban RH, Theise ND, eds. WHO classification of tumors of the digestive system; World Health Organization of Tumors. 4th ed. Lyon: IARC 225-227, 2010

4) Yamasaki S et al: Monotypic epithelioid angiomyolipoma of the liver. Histopathology 36(5): 451-456, 2000