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Disrupted cell adhesion but not proliferation mediates cyst formation in polycystic liver disease

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The pathogenesis of polycystic liver disease is not well understood. The putative function of the associated proteins, hepatocystin and Sec63p, do not give insight in their role in cystogenesis and their tissue-wide expression does not fit with the liver-specific phenotype of the disease. We designed this study with the specific aim to dissect whether pathways involved in polycystic kidney diseases are also implicated in polycystic liver disease. Therefore, we immunohistochemically stained cyst tissue specimen with antibodies directed against markers for apoptosis, proliferation, growth receptors, signaling, and adhesion. We analyzed genotyped polycystic liver disease cyst tissue ($n = 21$) compared with normal liver tissue ($n = 13$). None of the cysts showed proliferation of epithelial cells. In addition, anti-apoptosis marker Bcl-2 revealed a slight increase in expression, with variable increase of apoptosis marker active caspase 3. Growth factor receptors EGFR and c-erbB-2 were overexpressed and mislocalized. We found EGFR staining in the nuclei of cyst epithelial cells regardless of mutational state of the patient. Further, in hepatocystin-mutant polycystic liver disease patients, apical membranous staining of c-erbB-2 and adhesion markers MUC1 and CEA was lost and the proteins appeared to be retained in the cytoplasm of cyst epithelia. Finally, we found loss of adhesion molecules E-cadherin and Ep-CAM in cyst epithelium of all patients. Nevertheless, we observed normal β-catenin expression. Our results show that polycystic liver disease cystogenesis is different from renal cystogenesis. Polycystic liver disease involves overexpression of growth factor receptors and loss of adhesion. In contrast, proliferation or deregulated apoptosis do not seem to be implicated. Moreover, differential findings for *PRKCSH*- and *SEC63*-associated polycystic liver disease suggest a divergent mechanism for cystogenesis in these two groups.
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Patients suffering from polycystic liver disease (PCLD) develop numerous fluid filled cysts restricted to the liver.[1] So far, two genes, *PRKCSH* and *SEC63*, have been linked to PCLD by genome-wide linkage analyses and extensive sequencing.[2,3] The incriminated proteins, hepatocystin and Sec63p, respectively, are predicted to have their function in the glycosylation and transport of glycoproteins into and out of the endoplasmic reticulum (ER) (reviewed in Drenth *et al*[4]). Hepatocystin and Sec63p have a wide tissue expression,

but the phenotype of PCLD is restricted to the liver.[2,5–7] This paradox is not well understood and suggests that additional, liver specific, events occur that mediate cystogenesis.

Cystogenesis in other polycystic diseases such as autosomal dominant polycystic kidney disease (ADPKD) and autosomal recessive polycystic kidney disease (ARPKD) is studied to a greater extent. In these disorders cystogenesis is associated with deregulated apoptosis,[8,9] increased proliferation[10,11] and aberrant localization of growth factor receptors.[12–14] In addition, the Wnt signaling pathway, cell–cell adhesion and cell–matrix adhesion have been indicated to play a role in polycystic kidney diseases (PKDs).[15–21] Cell adhesion molecules not only mediate adhesion but also contribute to outside-in and inside-out cell signaling, which affects cell fate, differentiation and morphology (reviewed in Aplin *et al*[22]). To add another level of complexity,

all the processes mentioned above are intricately intertwined and interconnected.

In this study we set out to investigate whether the findings of the major pathways involved in renal cystogenesis can be extrapolated to PCLD. We hypothesized that cystogenesis in PCLD follows the same route as in ADPKD. Therefore, we analyzed the tissue expression of marker antigens of apoptosis, proliferation, cell growth, cell signaling and cell adhesion in cyst epithelium from PCLD patients.

Materials and methods

Tissues

Cyst tissue samples were collected from 21 PCLD patients who underwent laparoscopic cyst fenestration or liver transplantation due to PCLD (all female; median age at procedure, 44 years; range 35–62 years).[23] Mutational analysis showed that 16 patients carried a *PRKCSH* mutation, whereas 2 were *SEC63* mutants and 3 were wild type for both *PRKCSH* and *SEC63*. All mutations were found in a heterozygous state consistent with the autosomal dominant inheritance pattern of the disorder. Nonpathological tissue samples from 13 autopsy livers (4 male and 9 female patients; median age at death, 45 years; range 31–68 years) were used for control purposes. Autopsy patients died from causes other than liver disease or cancer. The study was performed according to the guidelines of the code for adequate use of secondary tissue (Version 2002, Federation of Medical Scientific Societies, www.fmwv.nl).

Immunohistochemistry

We prepared consecutive $4 \mu m$ paraffin sections, which we deparaffinized, rehydrated and blocked for endogenous peroxidase (30 min, 3% hydrogen peroxide in PBS). If necessary, antigen retrieval was performed by 10 min microwave exposure in 10 mM citrate buffer (pH 6.0) after which the slides were

allowed to cool down for at least 90 min. We used avidin/biotin-blocking kit (Vector Laboratories Inc., Burlingame, CA, USA) to block endogenous avidin and biotin and a 10 min preincubation with 20% normal horse serum to block a-specific binding sites. Primary antibodies (Table 1) were incubated overnight at 4°C followed by 30 min incubation with biotinylated horse anti-mouse antibody or goat anti-rabbit antibody (both from Vector Laboratories Inc.). Next, we tagged antibodies with avidin–biotin–peroxidase complex (Vector Laboratories Inc.) and chromagen 3,3′-diaminobenzidene with a hematoxylin counterstaining. Negative controls were produced by omitting the primary antibody.

Histological Evaluation

Sections were examined and photographed using a Zeiss Axioskop 2 FS plus microscope (Zeiss, Jena, Germany) and a ProgRes C10 plus digital camera with ProgRes Capture Pro 2.1 software (JenOptik, Jena, Germany). Immunohistochemistry results were classified as negative (−), weakly positive (+/−), positive (+), strongly positive (++), very intensely positive (+++). We distinguished clusters of moderately dilated bile duct structures as von Meyenburg complexes and conspicuously expanded bile ducts as cysts. However, most cyst tissue samples consisted of sections through cyst wall and lining cells were considered cyst epithelium. The proliferation index was defined as the percentage of Ki67/MIB-1-positive (+/−, +, ++ or +++) cells in the cyst epithelium.

Results

Proliferation and Apoptosis in PCLD

Increased proliferation and deregulated apoptosis are involved in ADPKD and ARPKD cystogenesis.[9,10,24] Therefore, we first examined proliferation markers in PCLD cyst tissue. Immunohistochemical staining of Ki67 revealed that in none of our patients' cyst

Table 1 Primary antibodies used for immunohistochemistry

Antigen	Antibody	Corporation	Antigen retrieval	Dilution
Active caspase 3	C92-605	BD Biosciences, San Jose, CA, USA	M	1:500
Bcl-2	Clone 124	DakoCytomation, Glostrup, Denmark	M	1:20
β-Catenin	Clone 14	BD Biosciences, San Jose, CA, USA	M	1:1000
CEA	B01-94-11M-P	BioGenex, San Ramon, CA, USA; monoclonal	—	1:320
CEA	A115	DakoCytomation, Glostrup, Denmark; polyclonal	—	1:2700
C-erbB-2	Clone CB11	BioGenex, San Ramon, CA, USA	M	1:20
E-cadherin	HECD-1	Takara Bio Inc., Otsu, Shiga, Japan	M	1:50
EGFR	Sc-03	Santa Cruz Biotechnology, Santa Cruz, CA, USA	M	1:100
Ep-CAM	Ber-EP4	DakoCytomation, Glostrup, Denmark	M	1:100
Ki67	MIB-1	DakoCytomation, Glostrup, Denmark	M	1:100
MUC1	Clone E29	DakoCytomation, Glostrup, Denmark	—	1:100

M, 10 min microwave exposure in 10 mM citrate buffer (pH 6.0); —, no antigen retrieval.

epithelia proliferation was increased compared to control liver bile ducts. In all patients the proliferation index was less than 1%, with only occasionally a positive cell in the cyst epithelium (Figure 1b). Lymphocytes present in the tissue samples showed positive staining and were regarded as an internal staining control.

Next, we studied apoptosis by assessing the expression of Bcl-2 (anti-apoptosis) and active caspase 3 (apoptosis). Compared to normal liver bile ducts we found that Bcl-2 staining was slightly elevated in cyst epithelia. In addition, some, but not all, bile ducts in cystic tissue had increased staining (Figure 1d). Finally, active caspase 3 expression was slightly elevated in cyst epithelia of some, but not all, PCLD tissue specimens regardless of genotype. Furthermore, active caspase 3 expression showed marked heterogeneity between different cysts in a single sample (Figure 1g–f). In controls, expression of Bcl-2 as well as active caspase 3 was absent.

Growth Factor Receptors are Expressed in Cyst Epithelia

We have determined the expression of two growth factor receptors in PCLD cyst epithelia: epidermal growth factor receptor (EGFR, c-erbB-1) and c-erbB-2 (Her2/neu). EGFR was strongly expressed in cyst epithelia and also hepatocytes were occasionally positive. Staining was seen often in the nucleus, and also in the cytoplasm of positive cells. Hepatocytes in control livers weakly expressed EGFR only in the cytoplasm. EGFR was negative in bile ducts in cyst tissue and in control liver.

C-erbB-2 expression showed divergent staining in patients with different mutations. Cyst epithelium of PRKCSH mutation carriers showed strong staining of c-erbB-2 in the cytoplasm of cyst epithelia. Patients, wild type for both genes or carrying a SEC63 mutation, showed expression of c-erbB-2 in some, but not all, cyst epithelia, and moreover, staining intensity was less explicit and mainly located on the apical surface. This staining resembled the normal bile duct staining in these patients. In contrast to the apical staining seen on all bile ducts in patient samples, only the large bile ducts in control liver samples showed positive apical staining for c-erbB-2. The smaller bile ducts were negative as were the hepatocytes. Representative images of the stainings are depicted in Figure 2.

Cell Signaling and Cell Adhesion

MUC1 is a glycoprotein active on the crossroads of many signaling pathways, from growthfactor signaling to Wnt signaling, cell adhesion and morphogenesis. We found MUC1 overexpression in the cytoplasm of the majority of cyst epithelia from patients with a PRKCSH mutation. The staining pattern matched exactly that of c-erbB-2 in these patients. In cyst epithelia from SEC63 mutation carriers and wild-type patients we also observed overexpression of MUC1. However, in most cyst epithelia staining showed a more apical concentration with weaker staining in the cytoplasm. This pattern resembled the apically centered expression in bile ducts seen in control liver (Figure 3a–c).

Next, we assayed the expression of CEA, a representative of the IgG-like adhesion molecules, using two different antibodies. As CEA is strongly glycosylated (28 potentially N-glycosylation sites), the epitope of the antibody raised against the protein backbone might be masked. In our study, both antibodies displayed the same staining pattern, yet the staining of the antibody raised against the fully glycosylated protein was more pronounced. CEA was strongly expressed on the bile canaliculi in normal liver and in patient cyst tissue. In contrast to negative staining of bile ducts, cyst epithelia showed strong cytoplasmic staining of CEA. In patients carrying a PRKCSH mutation, this staining pattern matched the patterns for MUC1 and c-erbB2. In these patients, cysts negative for MUC1 and c-erbB-2 were also negative for CEA. In SEC63-mutated patients or wild-type patients both positive cytoplasmic staining and negative staining was seen. Here, no correlation to other markers could be found (Figure 3d–f).

Further, we assayed the expression of E-cadherin as a representative of the cadherin family of adhesion molecules. We observed E-cadherin expression in hepatocytes and bile ducts of normal liver and in addition, in cyst epithelia of patient cyst tissue. Occasionally, minor focal loss of E-cadherin expression was seen in cyst epithelia of all patients without correlation to mutational state (Figure 3g–i). An absence of E-cadherin can cause disruption of the Wnt signaling pathway. To evaluate Wnt signaling involvement, we assessed the β-catenin expression in cyst epithelia. We found that β-catenin staining was strongly positive on membranes of all cyst epithelia, bile ducts and hepatocytes in normal liver and patient tissue. No translocation of β-catenin to the nucleus or cytoplasm was observed (Figure 3j–l). This implicates that in contrast to ADPKD cystogenesis, a disruption of Wnt signaling does not seem to be involved in PCLD cystogenesis.

Finally, the adhesion molecule Ep-CAM was stained because of its negative regulatory effect on adhesions mediated by the classic cadherins (including E-cadherin).[25] In concordance, we hypothesized that Ep-CAM would be (over)expressed in cyst epithelia that showed loss of E-cadherin. Surprisingly, Ep-CAM showed a more extensive loss than E-cadherin in cyst epithelia of all patients. Ep-CAM was expressed on the membrane of bile ducts in control liver and patient tissue (Figure 3m–o).

Table 2 summarizes the results of the immunohistochemical stainings of cyst epithelia compared
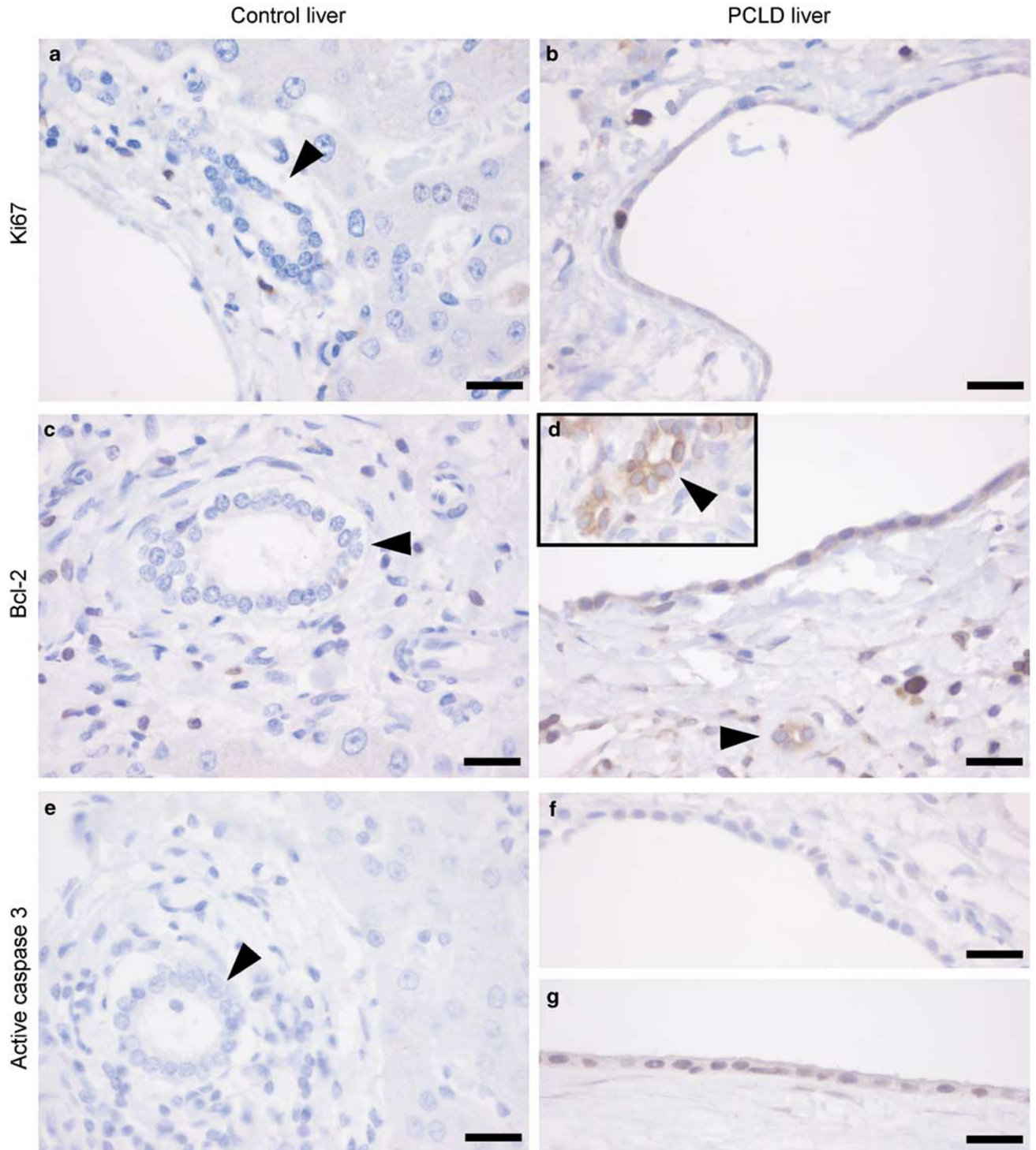
Figure 1 Expression of proliferation and apoptosis markers in control and PCLD liver. Immunohistochemical staining showed no increase in expression of proliferation marker Ki67 in PCLD liver compared to control liver (**a**, **b**). Expression of Bcl-2, an anti-apoptosis protein, is slightly elevated in cyst epithelia and bile ducts (inset) from PCLD patients (**c**, **d**). Finally, active caspase 3, an apoptosis marker, showed variable expression intensities within samples (**e–g**). We found no mutation dependent expression variation. Arrowheads indicate bile ducts and scale bars correspond to $50\,\mu$m.

to normal liver bile ducts. Most antibodies showed results similar for all patients regardless of mutational status. However, c-erbB-2, MUC1 and CEA showed differential expression in patients with a *PRKCSH* mutation.

Discussion

Here, we report that PCLD cyst tissue shows (1) normal proliferation, (2) slight increase in expression of anti- and proapoptosis factors, (3) increased
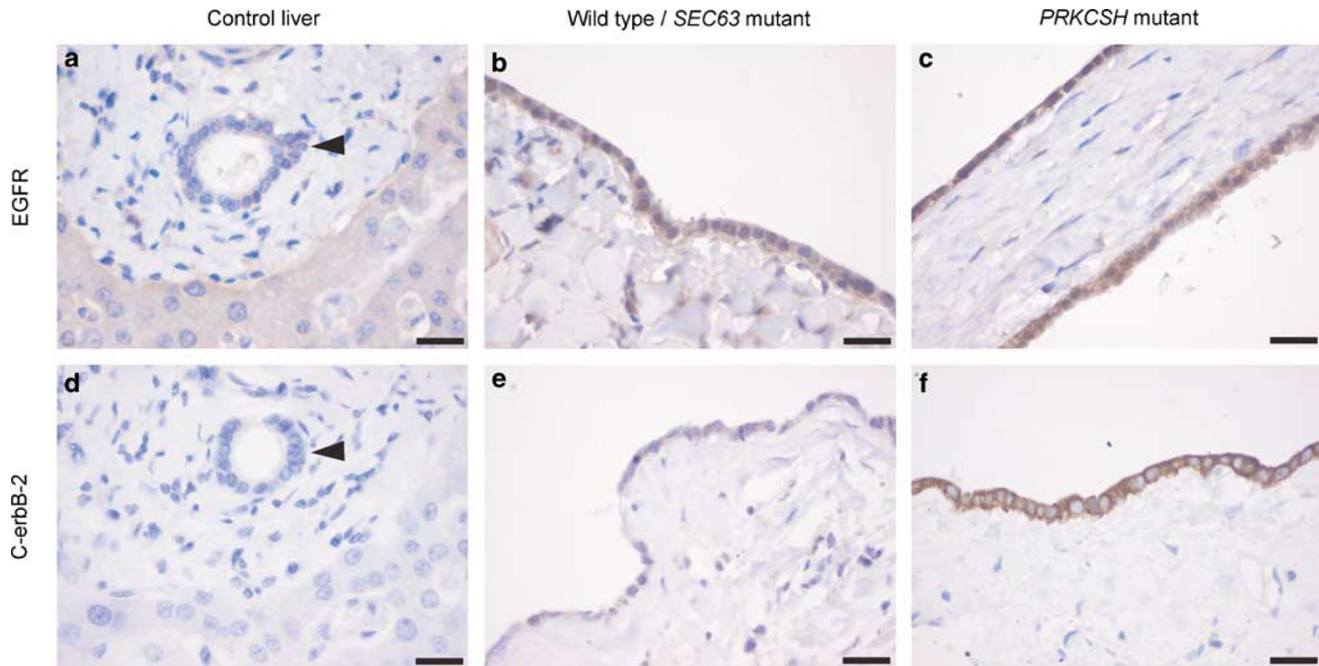
Figure 2 Expression of growth factor receptors, EGFR and c-erbB-2, in control and PCLD liver. We observed increased EGFR expression in nucleus and cytoplasm of PCLD cyst epithelial cells, regardless of mutational status (**a–c**). Patients carrying a *PRKCSH* mutation showed a distinct cytoplasmic overexpression of c-erbB-2. This is in contrast to a more variable c-erbB-2 expression in wild-type and *SEC63*-mutated patients (**d–f**). Arrowheads indicate bile ducts and scale bars correspond to 50 μm.

growth factor receptor expression, (4) loss of adhesion molecule expression and (5) mislocalization of c-erbB-2, MUC1 and CEA in *PRKCSH*-mutated cyst epithelia. The current results indicate that, similar to ADPKD, PCLD cystogenesis involves EGFR and c-erbB-2 overexpression. However, in contrast to the findings in ADPKD, PCLD cystogenesis does not seem to involve increased proliferation or (anti)-apoptosis.

Proliferation and Apoptosis

It has been suggested that deregulated proliferation and apoptosis play an important role in PKDs. Tissue sample proliferation indexes are increased.[10,11,26] In addition, several studies have shown that diverse factors, such as calcium, c-myc, laminin 5 and cAMP, increase proliferation in PKD cells.[27–31] Recently, Alvaro *et al*[11] found that in ADPKD liver cyst epithelia, proliferating cell nuclear antigen is highly expressed and that cyst fluid stimulates proliferation of cells derived from ADPKD liver cyst epithelia. In this study we used the MIB-1 antibody directed against Ki67 to determine the proliferation index in PCLD cyst tissue. This antibody stains cells in all phases but the G_0 (or rest) phase, in contrast to the commonly used staining for PCNA which is only expressed late in cell cycle phase G_1 and S phase.[32,33] Consequently MIB-1 gives a more valid impression of proliferation.

Our results show that proliferation is not increased in PCLD cyst epithelium compared to control liver bile ducts. The proliferation index was smaller than 1% in all patients studied. In addition, we found that all cysts (both large and small) were completely lined with cubic or flattened epithelium and we did not find any denuded basement membranes. Accordingly, as cyst size ranges from a few millimeters to several centimeters it can be argued that cyst epithelium has to proliferate to keep covering the entire cyst. Most cysts that were part of our sample were large and were located on the surface of the dorsal liver. The results suggest that these cysts expanded (passively) by fluid retention instead of actively proliferating to a large diameter. Finally, it can be argued that cysts grow either very slowly or by a nonlinear fashion, eg, in growth spurts. In summary, although some proliferation is needed to sustain the cyst coverage, (excessive) proliferation does not seem to be the main cause of cystogenesis in PCLD.

The role of apoptosis is ambivalent in PKDs. Both increased apoptosis and increased inhibition of apoptosis have been implicated in renal cystogenesis. In human ADPKD, increased Bcl-2 expression with normal levels of Bax seems to tip the balance to anti-apoptosis.[9] However, the same study detected increased apoptosis. Woo[24] found concordant results and reported that increased apoptosis causes loss of renal tissue and leads to renal dysfunction in ADPKD. Furthermore, the absence of the antiapoptotic protein Bcl-2 in *bcl-2*−/− mice leads to severe

PKD in animals as young as 10 days.[8] In addition, downregulation of Bcl-2 in the end stage of kidney development due to knockout of transcription factor AP-2β in *AP-2β−/−* mice also leads to massive apoptosis, polycystic kidneys and postnatal death.[34]
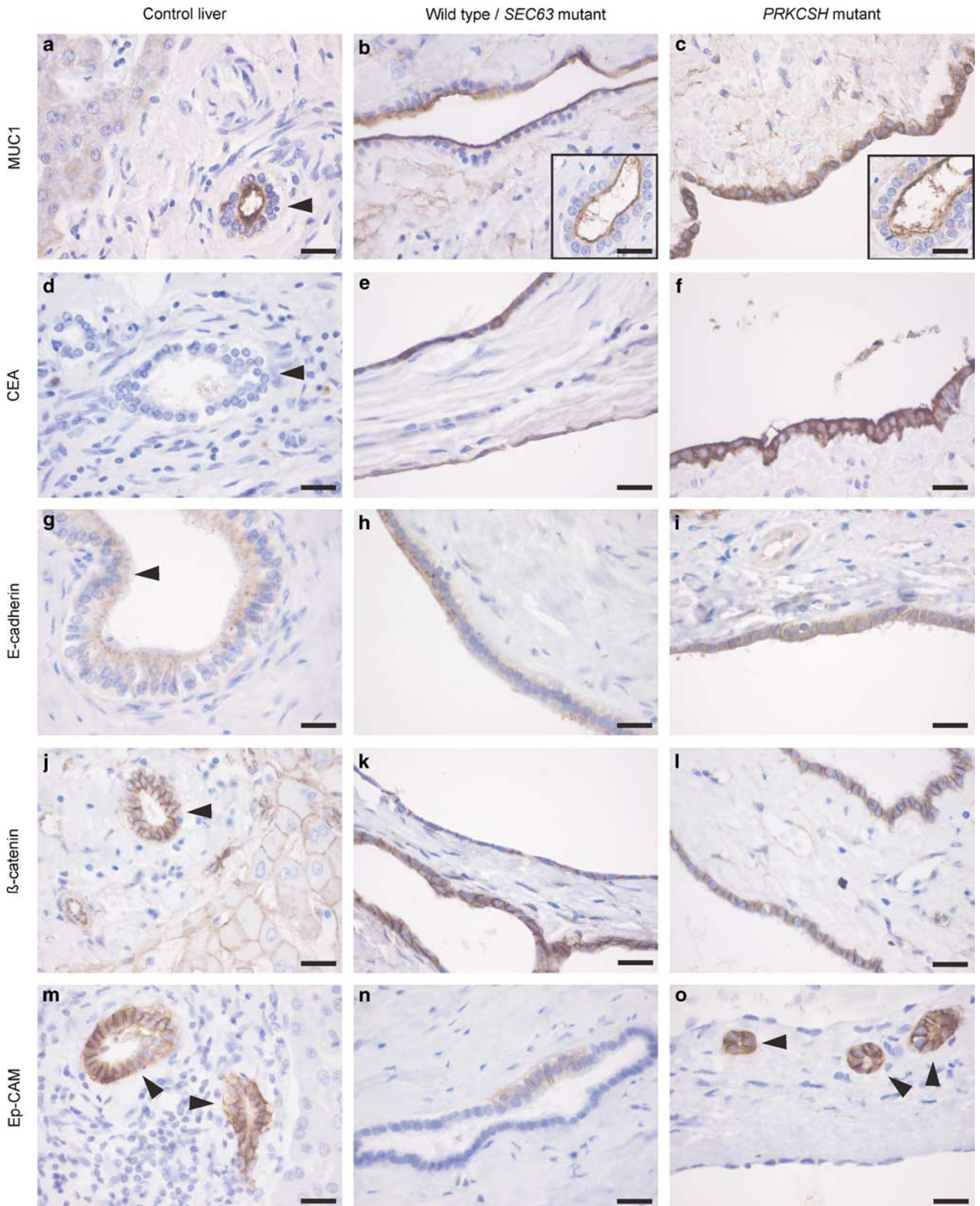
In this study, we found weak expression of Bcl-2 together with expression of active caspase 3 in some, but not all, cysts. It seems therefore that in some PCLD cysts the balance tips to anti-apoptosis (higher Bcl-2 and low active caspase 3) but in other cysts the balance seems to be in equilibrium (higher Bcl-2 together with high active caspase 3). This indicates that PCLD is a dynamic disease. The scored cysts are in different stages of growth and development, with some cysts degenerating and others developing.

Further, we found large numbers of bile ducts in cystic tissue with increased Bcl-2 staining (Figure 1d). Increased expression of Bcl-2 is found in reactive bile ducts in various cholestatic liver diseases.[35,36] Reactive bile ducts develop from proliferation of preexisting bile ductules and differ from normal bile ducts in their protein expression pattern. The existence of reactive bile ducts in cystic tissue would be a plausible explanation for the abundance of bile ducts found in some patients' tissues.

Growth Factors

Paradoxically, the overexpression of growth factor receptors EGFR and c-erbB-2 in PCLD cyst epithelia argues for presence of active proliferation. We found a nuclear staining pattern for EGFR in PCLD. This is reminiscent of highly proliferative or neoplastic tissues such as breast carcinomas. In the nucleus, EGFR can act as a transcription factor of cyclin D, iNOS and c-myb, expression of which contributes to increased proliferation.[37–39] Interestingly, translocation of EGFR is EGF-dependant and mediated by the Sec61 translocon.[40] This raises the question whether PCLD protein Sec63p, which is part of the Sec61 translocon, could also have a role in EGFR translocation.

In PKD cyst epithelia, both EGFR and c-erbB-2 are overexpressed and mislocalized to the apical surface, instead of normal basolateral expression on collecting tubules in the kidney.[12,14,41] Subsequently, overexpressed EGF accounts for an autocrine proliferative response.[12] In accordance, nonfunctional EGFR leads to less severe PKD in double mutant *orpk;wa2* mice[13] and selective inhibition of c-erbB-2 decreases cyst growth and results in improvement of kidney function in *PKD1* null mice.[14]

Mislocalization

In our study, three proteins, c-erbB-2, MUC1 and CEA, showed overexpression and mislocalization

restricted to or more pronounced in *PRKCSH*-mutated patients. Their staining pattern mirrored the staining pattern for hepatocystin.[42] Cysts devoid of hepatocystin expression showed the cytoplasmic staining pattern for c-erbB-2, MUC1 and CEA. The common denominator for these proteins is that they are all extensively *N*-glycosylated, a process mediated by hepatocystin. Defective glycosylation leads to misfolded glycoproteins resulting in retention in the ER or translocation out of the ER followed by degradation by the proteasome (reviewed in Ruddock and Molinari[43]). Destruction of the proteins mentioned above does not seem to be the case in our study as intense staining represents presence of protein. Additionally, *N*-glycosylation is important not only in protein folding but also in protein trafficking. For example, differences in glycan composition are found in secreted and membrane-bound MUC1.[44] MUC1 is a versatile protein interacting with, eg, EGFR, *β*-catenin, p53 and estrogen receptor-*α* (ERα), and also inhibiting proliferation and influencing cell–cell adhesion through its extraordinary length.[45–49] Intriguing is the fact that MUC1 stabilizes and stimulates ERα.[47] This is significant as hepatic cyst growth is promoted by estrogen supplementation and pregnancies.[1,50,51] Nevertheless, we did not find overexpression of ERα in PCLD tissues (data not shown). This suggests

Table 2 An overview of the results found in this study separated by genotype

Antibody	Mutation		
	PRKCSH	SEC63	WT
Ki67	N	N	N
Bcl-2	+/−	+/−	+/−
Active caspase 3	N/+	N/+	N/+
EGFR	+	+	+
C-erbB-2	+++[a]	N/+	N/+
MUC1	+++[a]	+	+
CEA	+++[a]	N/++	N/++
E-cadherin	F−	F−	F−
β-Catenin	N	N	N
Ep-CAM	F−−	F−−	F−−

[a]Corresponding pattern.
WT, wild type; N, normal expression; +/−, slight increase of expression; +, increased expression; +++ strong increase of expression; F−, focal loss; F−−, more extensive focal loss; N/+, normal expression in some cysts, increase of expression in other cysts; N/++, normal expression in some cysts, strong increase of expression in other cysts.

Figure 3 Immunohistochemical staining of adhesion molecules in control and PCLD liver. Staining of MUC1 revealed a slight increase in staining intensity in wild-type and *SEC63*-mutant patients. Staining was apically centered. In contrast, patients carrying a *PRKCSH* mutation showed strongly increased cytoplasmic expression in cyst epithelia. Bile ducts in all patients showed MUC1 staining on the apical membrane (insets) (**a–c**). We observed an increased expression of CEA in cyst epithelia. In addition, *PRKCSH*-mutant patients showed similar cytoplasmic overexpression as was seen in MUC1 and c-erbB-2 stainings (**d–f**). E-cadherin expression was focally lost in cyst epithelia regardless of mutational state (**g–i**). However, *β*-catenin expression was not lost and was solely found in membrane association (**j–l**). Finally, staining of Ep-CAM revealed extensive loss in all PCLD cyst epithelia (**m–o**). Arrowheads indicate bile ducts and scale bars correspond to 50 μm.

that the MUC1/ERα pathway is not responsible for increased cystogenesis in female PCLD patients.

The discrepancy between the results in *PRKCSH*-mutated patients and *SEC63*-mutated or wild-type patients indicate that PCLD cystogenesis might evolve through divergent pathways. A defect in hepatocystin seems to have effect on highly glycosylated proteins in addition to the general effects also seen in Sec63p mutants.

Adhesion

Finally, we turned our attention to the expression of adhesion molecules E-cadherin and Ep-CAM. The loss of both Ep-CAM and E-cadherin expression from PCLD cyst epithelia was surprising as expression of Ep-CAM is known to discard E-cadherin mediated cell–cell adhesions.[25,52] Our results indicate that loss of E-cadherin in PCLD cyst epithelia is not induced by Ep-CAM and that multiple adhesion processes are involved in PCLD cystogenesis.

Crucial for the normal functioning of E-cadherin is β-catenin.[53] However, β-catenin expression in PCLD cyst epithelia was unaltered compared to normal bile ducts. More specifically, we found no accumulation of β-catenin in the cytoplasm or nuclei. This suggests that the canonical Wnt signaling pathway is not active in PCLD cyst epithelia. This is in contrast with the findings in ADPKD and nephronophthisis, where cystic kidneys develop respectively due to the activation or the failure of inhibition of the canonical (β-catenin mediated) Wnt signaling pathway.[18,19,54] However, further research using more sensitive methods are warranted to confirm and specify our results.

Another arm of the Wnt signaling pathway, the noncanonical pathway or the planar cell polarity pathway, is also associated with ADPKD.[55] This pathway mediates cytoskeletal organization and cell polarity. Lengthening of tubules, a major component of kidney and liver development, involves lengthwise oriented cell proliferation. Loss of cell polarity undermines cell orientation and leads to cell division in planes other than the tubule axis, which can give rise to cystic structures. Our results cannot exclude involvement of this pathway in PCLD cystogenesis.

In summary, our results show that PCLD cystogenesis is different from cystic kidney diseases. PCLD cystogenesis involves overexpression of growth factor receptors and loss of adhesion, but no decrease or increase in apoptosis or proliferation. Moreover, differential findings for *PRKCSH*- and *SEC63*-associated PCLD suggest a divergent mechanism for cystogenesis in these two groups.

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Disclosure/conflict of interest

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