Overexpression of Aquaporin 1 on cysts of patients with polycystic liver disease

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ABSTRACT

Background and objective: Polycystic liver disease (PCLD) represents a group of genetic disorders that include autosomal dominant polycystic kidney disease (ADPKD) and isolated polycystic liver disease (iPCLD). There is currently no definitive treatment except for liver transplantation. The aim of this study was to assess the expression level of aquaporin 1 (AQP1) on the PCLD cysts with different sizes and provide the potential therapeutic target.

Methods: We collected 3 normal bile ducts, and recruited 8 patients with simple liver cyst disease, 24 patients with ADPKD, and 17 patients with iPCLD. AQP1 expression in different types of cyst walls and in normal bile ducts was detected using real time quantitative PCR, western blot and immunofluorescence staining. We also compared AQP1 expression levels in cysts of different sizes. Besides, ionic concentrations, pH and osmolality of cyst fluid were analyzed.

Results: The results showed that AQP1 expression in PCLD cysts was significantly higher than that in simple liver cysts and the normal bile ducts. In addition, a comparable increasing trend was found in cysts of smaller sizes to cysts of larger sizes. pH values, the sodium and chloride concentrations were higher in cyst fluid than that in the serum.

Conclusions: AQP1 was overexpressed in cystic cholangiocytes. A trend of increased AQP1 protein expression in correlation with the cyst size was also found. These observations offered a direction into the molecular mechanisms of cyst expansion and maybe provide new treatment strategies to reduce fluid secretion into liver cysts.

Key words: Aquaporin 1. Simple cystic liver disease. Autosomal dominant polycystic kidney disease. Isolated polycystic liver disease. Polycystic liver disease.

INTRODUCTION

Polycystic liver disease (PCLD) is defined as multiple fluid-filled cysts scattered throughout the liver parenchyma. In most patients, polycystic livers act as extra-kidney manifestations of autosomal dominant polycystic kidney disease (ADPKD), where polycystic kidneys are the primary phenotype. For the other patients, isolated polycystic liver disease (iPCLD) is characterized by the presence of multiple cysts in the livers in the absence of polycystic kidneys. ADPKD is genetically distinct from iPCLD: mutations in two genes (PKD1 and PKD2) are responsible for ADPKD, while three identified genes (PRKCSH, SEC63 and LRP5) and other unidentified genes cause iPCLD (1,2). Morphological studies reveal that liver cysts result from ductal-plate malformation (3). A hepatic cyst is considered to be a fluid-filled cavity lined with cholangiocytes. It is generally believed that liver cyst enlargement is caused by intensive proliferation in the epithelium in the cyst lining, neovascularization, fluid secretion into the cysts, and remodeling of the extracellular matrix surrounding the cysts (4,5).

Aquaporins (AQPs) are a group of proteins discovered in the early 1990s that mediate the bidirectional passive movement of water molecules across epithelial cells in response to osmotic gradients established by ions and solutes. The water permeability through AQPs is 5- to 50-fold greater than that through the lipid bilayer (6). There are several types of AQPs expressed on the apical and basolateral cholangiocyte membrane domains and on intracellular vesicles: AQP0, AQP1, AQP4, AQP5, AQP8, AQP9 and AQP11, which are increasingly recognized as important in water transport by biliary epithelia (7). As previously demonstrated in polycystic kidney rat model, overexpression of AQP1, the chloride channel CFTR, and the anion exchanger AE2 is associated with hepatic cystogenesis (8). Expression, localization, and function of the AQPs in human ADPKD kidney cysts have been investigated (9-12). However, there are few studies on AQPs expression and direct functional studies demonstrating AQPs-mediated water transport in human PCLD liver cysts.
Although there are medical treatments and various surgical therapies for PCLD, they are also associated with morbidity and mortality to some extent and a considerable risk of recurrence. The only definitive treatment in PCLD patients remains liver transplantation (13,14). Therefore, it is necessary to search for new therapeutic targets for PCLD. We hypothesized that altered expression of the functionally related protein \( i.e. \) AQPI could contribute to hepatic cystogenesis and enlargement of cysts. Thus, AQPI expression in the normal bile duct, simple liver cysts, ADPKD cysts and iPCLD cysts was investigated using real-time quantitative PCR (RT-qPCR), western blot, and immunofluorescence staining. The composition, pH and osmolality of cyst fluid were also analyzed.

**MATERIALS AND METHODS**

**Data acquisition**

Detailed information was collected to confirm liver cyst types in each patient who was assessed for eligibility. Key elements included age, sex, family history, medical history, estrogen exposure history, date of diagnosis, symptoms at presentation, findings on physical examination, abdominal radiological data, complications, therapy and outcomes. The number and size of liver cysts were determined using ultrasound, CT or MRI. Laboratory data included creatinine, urea nitrogen, aspartate aminotransferase (AST), alanine aminotransferase (ALT), alkaline phosphatase (ALP), \( \gamma \)-glutamyl transferase (GGT), albumin and total bilirubin.

**Diagnosis and sampling**

Simple cystic liver disease (SCLD) was defined as having no more than two liver cysts and no positive family history. ADPKD diagnosis was based on the Ravine criteria (15). Isolated PCLD is characterized by the presence of multiple liver cysts of biliary epithelial origin without significant cystic involvement of other organs. Sporadic cases of PCLD were defined as having more than 20 cysts throughout the liver. In patients with a positive family history, diagnosis was made when more than four liver cysts were present. Exclusion criteria included the presence of incomplete data, pathological diagnosis of adenoma and amount of liver tissue adhering to the cyst walls based on pathological findings and recent liver surgery.

The research project conforms to the provisions of the Declaration of Helsinki (as revised in Seoul, Korea, October 2008). Written informed consent and the approval of Jilin University Ethics Committee were obtained before sample collection. A total of 62 patients were recruited from the First Bethune Hospital of Jilin University (Changchun, China) from March 2012 to May 2015, and 49 who met the criteria were enrolled. Cyst wall tissue and cyst fluid were acquired from three recipients, which represented normal bile ducts, were acquired from three recipients with post-hepatitis cirrhosis and one female individual with a medicamentous liver lesion; mean age 46.5 ± 7.3 years old).

**Real-time quantitative PCR**

Total RNA was isolated from snap-frozen liver cysts using the TriSure kit (Bioline, London, UK) and quantified using spectrophotometric absorbance at 260nm. cDNA was synthesized using FastQuant RT kit (Tiangen Biotech, Beijing, China). Each reaction was performed in triplicate using a final reaction volume of 20μL. Primers for AQPI (forward 5'-CAGCCCAAGGACAGTTCA-GAGA-3', reverse 5'-GGCTAAGTGCACAGTGGGGA-3') and -actin (forward 5'-GGAGATTACTGCCCTGGCCTCA-3', reverse 5'-GACTCATCGTACTCCTGCTTG CGTG-3') were synthesized by TaKaRa Biotechnology Co., Ltd. (Dalian, China). All samples were normalized to \( \beta \)-actin mRNA levels. Data analysis was performed using the 2\(^{-\Delta\Delta C_{t}} \) method.

**Western blot**

Total protein from the cyst walls and bile ducts was extracted using mammalian protein extraction reagent (M-PER; Thermo Scientific) and protein concentrations were determined with the bicinchoninic acid protein assay (Pierce Chemical). Rabbit anti-human AQPI mAb (Abcam, England) diluted 1:2000 were used in this experiment, along with horseradish peroxidas-conjugated mouse anti-rabbit immunoglobulin diluted 1:2000 (Bioss, Beijing, China). Protein bands were detected using an enhanced chemiluminescence detection system (ECL Plus, Amershams Biosciences) and a gel imaging system (ChemiDocXRS+, USA).

**Immunofluorescence staining**

Formalin-fixed, paraffin-embedded tissue slices (4 μm) were immunostained as described (10) to visualize the localization and distribution of AQPI. After incubated overnight at 4 °C with rabbit anti-human AQPI monoclonal antibody (Abcam, England), 1:300 diluted, the tissue sections were then incubated for 1h in fluorescein conjugated mouse anti-rabbit immunoglobulin diluted 1:2000 (Bioss, Beijing, China). Protein bands were detected using an enhanced chemiluminescence detection system (ECL Plus, Amershams Biosciences) and a gel imaging system (ChemiDocXRS+, USA).

**Analysis of cyst fluid composition**

Before fenestration or/and hepatic resection for liver cysts, surgeons punctured the liver cysts using long and thin puncture needles, and aspirated the cyst fluid using 50ml syringes. Ionic concentrations of sodium, potassium, calcium, and chloride in an aliquot of cystic fluid were promptly analyzed (Vitro 350, Johnson & Johnson, USA). The pH and osmolality of each solution was verified with a pH/mV electrometer (PB-10, Sartorius, Germany) and a freezing-point osmometer (Osmomat 030, Gonotec, Germany), respectively.
Statistical analysis

All of the data was analyzed using SPSS 16.0 statistical software (SPSS Inc., Chicago, IL, USA). Results are presented as the mean ± standard deviations in all tables. Categorical data analysis was conducted using the Pearson test, χ² test, or Fisher exact test, and continuous data were analyzed using the Student t test or the Mann-Whitney U test, as appropriate. All P values were two-tailed, and p < 0.05 was accepted as being statistically significant.

RESULTS

Clinical characteristics

PCLD cysts have radiological characteristics identical to simple liver cysts, except that there are more cysts in PCLD (Fig. 1). The distinction between iPCLD and ADPKD relies on the number of renal cysts, age at presentation and family history. Women presented a large and symptomatic cystic liver more frequently than men for both ADPKD and iPCLD (Table I). Logistic regression analysis showed that there is a mild positive correlation between the number of liver cysts and the time of estrogen exposure in PCLD patients (r = 0.67, p < 0.05, data not shown). Compared with iPCLD, ADPKD patients were significantly more likely to have a positive family history, but fewer cysts that are smaller in size.

Liver cysts usually have an asymptomatic course. Despite large cysts and decreased volume of normal liver tissue, the physiological function of the liver is almost always normal, as shown in the laboratory results. Similar to other studies, patients with PCLD had slightly higher levels of serum ALP and GGT than healthy people or patients with SCLD (16). Patients with ADPKD mostly had elevated serum creatinine and urea nitrogen that was caused by larger renal cysts. Symptoms caused by the effect of the cyst mass mainly include abdominal pain, abdominal distention, dyspepsia, dyspnea, and, rarely, early satiety, back pain, obstructive jaundice, portal hypertension, transudative and exudative ascites, and Budd-Chiari syndrome. There was no significant difference in the distribution of these symptoms between the three types of cystic liver diseases.

Upregulation of AQP1 expression in PCLD cysts compared with the normal bile duct and simple liver cysts

Normal bile ducts and simple liver cysts were used as the control group in the AQP1 expression analysis. All results were normalized to β-actin and the fold changes are reported relative to the normal bile duct. For AQP1 mRNA expression, both simple liver cysts and PCLD cysts were significantly higher than normal bile ducts. iPCLD cysts represented the largest fold change, approximately 20-fold. Moreover, AQP1 mRNA expression in simple liver cysts was significantly lower than in PCLD cysts, and that in iPCLD cysts was significantly higher than in ADPKD cysts (Fig. 2A).
Western blot analysis showed that AQP1 was significantly enriched in liver cysts, compared with normal bile ducts (Fig. 3 A and B). The pattern of different AQP1 expression levels in simple liver cysts, ADPKD cysts and iPCLD cysts was consistent with the result from RT-qPCR (Fig. 2A).

As shown in figure 4, AQP1 was detected in the cyst-lining cells located on the inner surface of the cyst wall and the bile duct epithelial cells located on the body of cyst wall for the three types of liver cysts. In normal bile ducts, weak diffuse AQP1 expression was detectable in cholangiocytes, localized mainly at the cell membrane. The fluorescence intensity of liver cysts was significantly higher than that of a normal bile duct. Compared with simple liver cysts, higher expression was more commonly observed for both ADPKD cysts and iPCLD cysts. In addition, we measured the longest outer diameter for each bile duct and the number of capillaries present in different liver cyst walls in five random visual fields for each cyst wall, at a magnification ×100. There was no significant difference in the number of cholangiocytes and capillaries between simple liver cysts, ADPKD cysts and iPCLD cysts.

### Differences of AQP1 expression in PCLD cysts of different sizes

To detect changes in AQP1 expression in the progression of PCLD cysts, we compared the largest with the smallest liver cysts that were removed from each patient; the gap of diameter was more than 5cm. Within each ADPKD (n = 5) or iPCLD (n = 6) patient, the mRNA expression pattern of AQP1 in cyst walls showed a significant increasing trend from smaller cysts to larger cysts (Fig. 2 B and C). Western blot analysis of AQP1 in PCLD cysts of different sizes confirmed the same trend in both ADPKD and iPCLD patients (Fig. 3 C and D).
The genetic defect resulting in PCLD has been identified, but the molecular pathogenesis of liver cyst growth remains unclear. Researches have demonstrated that abnormalities in cholangiocyte proliferation and enhanced fluid secretion are key factors in the pathophysiology (17,18). Fluid secretion into liver cysts promotes stretching of the epithelium lining the cyst, causing the release of cytokines into the cyst fluid, which then signal an acceleration of cyst growth (19). In some research on ADPKD, it has been demonstrated that AQPs play specific roles in epithelial cells that line various segments of the kidney (10). In cholangiocytes that line the intrahepatic bile ducts, a transcellular pathway plays a key role in transepithelial water movement, rather than a paracellular pathway (20). AQPs also help to mediate water movement (7). Thus, we tested the alteration of AQP1 expression on different kinds of liver cysts with different sizes and provided a potential contributor to the development and growth of PCLD cysts.

ADPKD affects up to 0.2% of the general population (2), while iPCLD has a morbidity of less than 0.01% (21). All PCLD liver cysts arise from progressive dilatation of abnormal ductules that display the same epithelium and structures as functioning cholangiocytes (22,23), but patients with iPCLD tend to have larger sized cysts (24). The annual growth of PCLD livers is in the range of 0.9-3.2% of the initial hepatic volume and the speed becomes faster and faster (20). Most of the patients with PCLD are asymptomatic. However, a minority of patients (20%) have expansion of liver cysts that cause serious abdominal symptoms (1,2), and less than 5% of patients have acute complications (24). These symptoms tend to be equally distributed in ADPKD and iPCLD. Normal liver function

Cystic fluid analysis

A total of 91 liver cyst fluid samples were included in this study (8 patients with SCLD, 8 samples; 24 ADPKD patients, 45 samples; 17 iPCLD patients, 38 samples). The concentration of potassium and the osmolality of the cyst fluid were within the range of normal serum values, but they showed great variability. As table II shows, glucose in cyst fluid was significantly lower than the normal serum value (p < 0.001). However, pH values and the sodium and chloride concentrations were higher in cyst fluid than that in the serum (p < 0.01). There were large differences between patients and also between individual cysts obtained from the same patient. We also observed no significant differences in the contents of liver cyst fluid among simple liver cysts, ADPKD cysts, and iPCLD cysts.
is usually maintained even at the end stages of iPCLD, while ADPKD patients generally present with defective renal function. For symptomatic patients, all the currently available surgical options except liver transplantation have been performed only to alleviate the abdominal symptoms by decreasing cyst volume, but this does not change the natural course of the disease. As recently reported, somatostatin analogs have an apparent beneficial effect on slowing the progressive increase in liver volume, but not all patients with PCLD respond to treatment (14). Therefore, in an era of organ shortage, other therapeutic targets to prevent the speed of cyst growth, recurrence, and even cystogenesis need to be developed.

AQP1 is expressed in structures such as the biliary epithelium, blood vessels, red cells and liver cells. Therefore, various measures were taken to exclude the effect of other tissues or cells on AQP1 expression in the epithelium lining the liver cysts, which included washing away the red cells from the cyst walls immediately after the surgical removal, and not including the cyst walls that were adhered to the liver tissue mount, based on pathological findings. Moreover, there is no significant difference in the number of cholangiocytes and capillaries among simple liver cysts, ADPKD cysts and iPCLD cysts (data not shown). In the present study, the principal finding was that elevated constitutive AQP1 expression was found on liver cyst walls. AQP1 expression significantly increased, indicating that AQP1 might provide a faster route for osmotic water transportation across the epithelium lining the cyst. In contrast, our previous study on AQP9 showed that there was no significant expressional difference in different cyst types (data not shown). This suggested that AQP1 was an important contributor to water secretion in the epithelium lining the liver cyst. However, the involvement of other AQPs that may compensate or alternative AQPs that may be involved in cholangiocyte fluid secretion cannot be discounted. The

**Table II. Cyst fluid composition, pH and osmolality**

<table>
<thead>
<tr>
<th></th>
<th>SCLD</th>
<th>ADPKD</th>
<th>iPCLD</th>
<th>Plasma (Ref.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Na⁺ (mmol/L)</td>
<td>149.2 ± 3.4</td>
<td>148.1 ± 7.3</td>
<td>148.5 ± 5.9</td>
<td>137-147</td>
</tr>
<tr>
<td>K⁺ (mmol/L)</td>
<td>3.9 ± 0.3</td>
<td>4.2 ± 0.9</td>
<td>4.1 ± 0.7</td>
<td>3.5-5.3</td>
</tr>
<tr>
<td>Ca²⁺ (mmol/L)</td>
<td>0.8 ± 0.2</td>
<td>1.1 ± 0.5</td>
<td>1.2 ± 0.4</td>
<td>2.1-2.5</td>
</tr>
<tr>
<td>Cl⁻ (mmol/L)</td>
<td>120.3 ± 3.5</td>
<td>123.2 ± 4.6</td>
<td>122.8 ± 3.3</td>
<td>99-110</td>
</tr>
<tr>
<td>Glucose (mmol/L)</td>
<td>0.21 ± 0.06</td>
<td>1.26 ± 0.53</td>
<td>0.87 ± 0.41</td>
<td>4.1-5.9</td>
</tr>
<tr>
<td>pH</td>
<td>7.91 ± 0.14</td>
<td>7.61 ± 0.16</td>
<td>8.06 ± 0.28</td>
<td>7.35-7.45</td>
</tr>
<tr>
<td>Osmolality (mOsmol/kg)</td>
<td>274.5 ± 6.7</td>
<td>286.0 ± 14.3</td>
<td>294.7 ± 16.3</td>
<td>275-295</td>
</tr>
</tbody>
</table>

SCLD: Simple cystic liver disease; ADPKD: Autosomal dominant polycystic kidney disease; iPCLD: Isolated polycystic liver disease.

**Fig. 4. Immunolocalization and distribution of staining intensity of AQP1 in normal bile ducts, simple liver cysts and PCLD cysts. AQP1 was detected in the bile duct epithelium (A and E), the cyst-lining cells located on the inner surface of the cyst wall (B-D), and the bile duct epithelial cells located on the body of cyst wall for the three types of liver cysts (F-H). AQP1 expression on bile duct epithelium and cyst-lining cells is strongly upregulated in PCLD cysts, compared with normal bile duct and simple liver cysts. Moreover, AQP1 staining intensity in iPCLD cysts was somewhat stronger than that in ADPKD cysts. Original magnifications: (A-H) x200. Scale bar, 20 μm (*lumen of bile duct or liver cyst).**
difference in AQP1 expression between ADPKD cysts and iPCLD cysts was similar to the mean cysts sizes. This heterogeneity of protein expression in PCLD cysts and SCLD cysts suggests that more specific mechanisms may exist in protein trafficking, such as cross reaction with the protein encoded by the mutant gene and the function of specific cytokines. Without regard to other AQPs, the comparable increasing AQP1 expression trend found in cysts of smaller sizes to cysts of larger sizes could result in fluid secretion of higher speed in larger cysts, which is in consistent with the increasing cyst enlarging speed.

In PCLD, several altered intracellular signaling pathways together with a number of specific cytokines and growth factors are responsible for hyperproliferation and hypersecretion of the cholangiocytes (25-27). In addition to the ions involved, we suggest that specific water channel expression might be an additional feature related to fluid permeability associated with net fluid secretion into the cystic lumen. Moreover, fluid secretion into liver cysts promotes stretching of the cyst lining epithelium, and the latter could result in the release of cytokines into the cyst fluid, which then signals an acceleration of cyst growth (28). In this study, the presence of AQP1 at the apical and basolateral cholangiocyte domains, together with the comparable increasing trend from cysts of smaller sizes to cysts of larger sizes, suggested that AQP1 is likely an important mediator of water movement through cholangiocytes during iPCLD cyst expansion. The difference in AQP1 expression in ADPKD and iPCLD cysts was consistent with this trend. However, previous studies on ADPKD kidney cysts showed opposite results (11). To our knowledge, the discrepancy was mainly a result of the varying AQP1 expression and localization in different tissues/cells. Quantitative analysis was also performed in ADPKD kidneys rather than the kidney cyst walls. Further, there was also a possibility that AQP1 was not the dominant water channel that was responsible for expansion of kidney cysts.

The cystic fibrosis transmembrane conductance regulator protein, a cAMP-regulated chloride channel expressed in the apical membrane of epithelial cells lining the cyst, is believed to be the principal route for chloride entry into cysts, and sodium is secondarily driven across the epithelium into the cystic lumen by the inner and outer lumen potential. In response to osmotic gradients induced by sodium influx, water is transported into the cyst through AQPs that are expressed on the epithelial cells lining the cyst. Furthermore, the increasing intracapsular pressure caused by accumulation of cyst fluid leads to compensatory hyperplasia of epithelial cells. AE2, a Cl/HCO3 exchange, also participates in this process (8). This type of mechanism is sufficient to explain the composition, pH, and osmolality. Thus, the rapid movement of plasma water across both the apical and basolateral cholangiocyte membranes would allow relative iso-osmolar status of the cell to be maintained. In turn, this status may facilitate ion and water movement from the basolateral to the apical membrane and may account for enhanced fluid secretion into the cyst lumen (29).

In conclusion, several independent and complementary biochemical and morphologic approaches have shown that AQP1 was overexpressed on PCLD cysts. These observations provide a direction into the molecular mechanisms of cyst expansion and suggest a new potential target to reduce fluid secretion into liver cysts. Further functional research is needed to confirm the precise mechanism of the gene mutations, as described above, which affect AQP1 expression in the epithelial cyst lining and eventually lead to PCLD cyst expansion.

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