Secondary, Somatic Mutations Might Promote Cyst Formation in Patients With Autosomal Dominant Polycystic Liver Disease

MANOE J. JANSSEN,* ESMÉ WAANDERS,*,‡ RENÉ H. M. TE MORSCHE,* RUOYU XING,* HENRY B. P. M. DIJKMAN,§ JANNES WOUDENBERG,* and JOOST P. H. DRENTH*

*Departments of *Gastroenterology and Hepatology, and ‡Human Genetics, Institute for Genetic and Metabolic Disease, Radboud University Nijmegen Medical Centre, Nijmegen; §Department of Pathology, Nijmegen Centre for Molecular Life Sciences, Radbound University Nijmegen, Nijmegen, The Netherlands*

BACKGROUND & AIMS: Heterozygous germline mutations in *PRKCSH* cause autosomal dominant polycystic liver disease (PCLD), but it is not clear how they lead to cyst formation. We investigated whether mutations in cyst epithelial cells and corresponding loss of the *PRKCSH* gene product (hepatocystin) contributed to cyst development. **METHODS:** Liver cyst material was collected through laparoscopic cyst fenestration from 8 patients with PCLD who had a heterozygous germline mutation in *PRKCSH*. Tissue sections from 71 cysts (2–14 per patient) were obtained for hepatocystin staining and mutation analysis. Cyst epithelium was acquired using laser microdissection; DNA was isolated and analyzed for loss of heterozygosity (LOH) and somatic mutations using restriction analysis and sequencing. Common single nucleotide polymorphisms (SNPs) in a 70-kilobase region surrounding the germline mutation were used to determine variations in the genomic region with LOH. **RESULTS:** The wild-type allele of *PRKCSH* was lost (LOH) in 76% of cysts (54/71). Hepatocystin was not detected in cyst epithelia with LOH, whereas heterozygous cysts still expressed hepatocystin. The variation observed in the LOH region analysis indicates that cysts develop independently. We also detected somatic mutations in *PRKCSH* in 17% (2/12) of the cysts without LOH. Trans-heterozygous mutations in SEC63 were not observed. **CONCLUSIONS: Among patients with PCLD who have a heterozygous germline mutation in *PRKCSH*, we found secondary, somatic mutations (second hits) in more than 76% of the liver cyst epithelia. PCLD is recessive at the cellular level, and loss of functional *PRKCSH* is an important step in cystogenesis.**

Keywords: Cholangiocytes; Genetic Analysis; Pathogenesis; Liver Disease.

Autosomal dominant polycystic liver disease (PCLD) is a genetic condition characterized by the presence of multiple fluid-filled hepatic cysts. These cysts cause a grossly enlarged liver with displacement of other abdominal organs. Patients experience abdominal pain, nausea, anorexia, and shortness of breath.[1] PCLD is a dominantly inherited disorder and so far 2 genes, *PRKCSH*[2,3] and *SEC63*,[4] have been associated with the disease. These genes encode for hepatocystin and SEC63, respectively, which are both endoplasmic reticulum (ER) resident proteins, widely expressed in most cell types including hepatocytes and cholangiocytes.[5] Hepatocystin is assembled as a heterodimer complex, which acts to achieve proper topology and folding of membrane and secreted glycoproteins in the ER. SEC63 is believed to play a role in protein transport across the ER membrane.[6] How these proteins are involved in the development of polycystic livers remains unknown, and a better understanding of the initiating event underlying cyst formation would provide valuable insight into the pathogenesis.

Under physiologic conditions, maturation of the ductal plate leads to development of normal bile ducts. In PCLD, dense complexes of intralobular bile ductules remain intact and develop into cysts by disconnecting from the biliary tree through a process of differentiation and proliferation.[7] It has been speculated that cyst formation results from a focal process involving only a subset of cells. This theory implies that each cyst starts with a defining genetic event.[8]

All known mutations in PCLD are heterozygous and affect only one allele of the gene while the other allele is normal (wild type).[9] Furthermore, in liver tissue sections from patients with a *PRKCSH* germline mutation, hepatocystin is normally expressed in both hepatocytes and cholangiocytes from normal bile ducts. In contrast, hepatocystin appears to be lacking from the cells most relevant to PCLD: cholangiocytes from liver cyst epithelium.[10] It is not known why cyst epithelium does not express hepatocystin and how this relates to the heterozygous state of the germline mutation in PCLD. Abnormal cholangiocyte growth and cyst formation could result from a dominant negative effect of the mutant *PRKCSH* on the expression or function of the wild-type allele in this tissue. Alternatively, the wild-type *PRKCSH* allele could be lost through somatic genomic mutations, consistent with the so-called 2-hit model.[11] To delineate the molecular mechanism underlining cyst formation in PCLD, we isolated cyst epithelium from patients with a *PRKCSH* germline mutation to identify possible somatic mutations. Our data

Abbreviations used in this paper: ADPKD, autosomal dominant polycystic kidney disease; ER, endoplasmic reticulum; LOH, loss of heterozygosity; PCLD, polycystic liver disease; SNP, single nucleotide polymorphism.

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suggests that the 2-hit model holds true for the primary manifestation of human PCLD and supports the hypothesis that PCLD is recessive on a cellular level.

Materials and Methods

Patient Material

We obtained whole blood and liver tissue samples from 8 patients with PCLD who underwent laparoscopic cyst fenestration because of severe symptoms.[12] All patients were female, and age at the time of surgery ranged between 35 and 54 years of age (median age, 41 years). We used formalin-fixed, paraffin-embedded liver tissue samples that had routinely been collected for pathologic examination (n = 22 from 3 patients) or fresh tissue samples that had been snap frozen immediately after excision and stored at −80°C until analysis (n = 49 from 5 patients). All samples were collected with appropriate ethics approval, and written informed consent for the use of secondary tissue was obtained from all patients.

Genotyping

We screened patient DNA from whole blood for germline mutations in *PRKCSH* and *SEC63* using direct sequencing as described previously.[9] In brief, DNA from whole blood was isolated using the Puregene DNA Isolation Kit (Gentra Systems, Minneapolis, MN) and stored at 4°C. Exons and flanking intronic sequences were amplified using polymerase chain reaction with specific primers (Supplementary Table 1). The amplified fragments were purified (QIAEXII Gel Extraction Kit; Qiagen, Hilden, Germany) and sequenced with the BigDye Terminator Kit and ABI3730 capillary sequencer (Perkin-Elmer Applied Biosystems, Boston, MA).

Laser Microdissection

Tissue sections (10 μm) from frozen or formalin-fixed, paraffin-embedded liver samples were mounted on cross-linked PEN membrane slides (Leica Microsystems GmbH, Wetzlar, Germany), stained with Mayer's hematoxylin (1 minute), and rinsed in tap water. Paraffin-embedded sections were deparaffinized using xylene and ethanol before hematoxylin nuclear stain. Specific isolation of the cyst epithelial cells (300 to 2000 cells/sample) was performed using a Leica Laser Microdissection System (LMD 6000, Leica Microsystems GmbH) equipped with an UV laser (Leica Microsystems GmbH).[13] For each patient, we dissected liver cells (hepatocytes and other noncyst epithelial cells) to serve as a control sample.

DNA Isolation

DNA from the dissected cells was isolated using the QIAamp DNA Micro Kit (Qiagen) according to instructions and with the use of carrier RNA. To increase the DNA yield and quality from formalin-fixed tissue samples, we made the following adjustments: samples were digested at 56°C for 2 days with occasional agitation, proteinase K was added in 2 steps (5 μL on day 1 and 5 μL on day 2), and after digestion and addition of buffer ATL, samples were incubated at 90°C for 1 hour to promote reverse cross-linking of the DNA.

Loss of Heterozygosity Analysis in Cyst Epithelium

Loss of heterozygosity (LOH) was analyzed in all samples by amplifying the genomic region containing the germline mutation followed by digestion or sequencing (Supplementary Table 2).

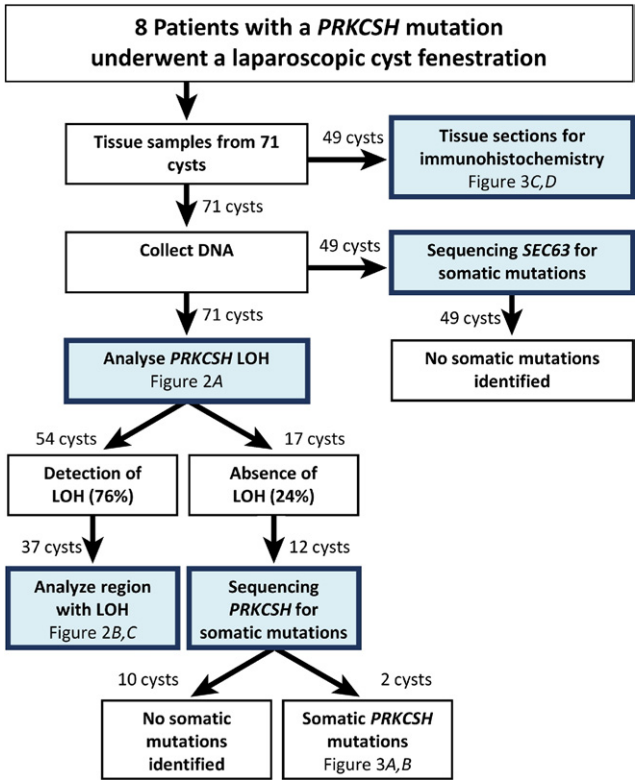

Figure 1. Somatic mutation analysis. Flow chart of sample collection and analysis of 71 cysts from 8 patients with PCLD who have a *PRKCSH* germline mutation. Cyst epithelial cells were isolated from all cysts with laser microdissection for subsequent DNA isolation and LOH analysis. In addition, samples from frozen tissue (49 cysts) were used for immunohistochemical analysis, somatic mutation analysis of *PRKCSH* and *SEC63*, and LOH region analysis.

Somatic Mutations and Region Analysis

We conducted the following analyses on DNA isolated from frozen tissue samples (Figure 1).

1. *PRKCSH* sequencing. In cysts without LOH, all *PRKCSH* coding exons and flanking intronic sequences were sequenced (as described for genotyping) to detect somatic mutations in DNA from the cysts. DNA isolated from whole blood of the patient was used as a reference sample.
2. To yield enough material for the LOH region analysis and *SEC63* sequencing, cyst epithelial DNA was amplified using a commercially available whole genome amplification kit (GenomePlex WGA; Sigma, St Louis, MO) and purified (GenElute PCR Clean-Up Kit; Sigma) before analysis. Sample amplification was performed in duplicate to control for any mutations resulting from the whole genome amplification procedure.
3. LOH region analysis. For cysts displaying LOH, we used 5 common (no pathogenic) single nucleotide polymorphisms (SNPs) to analyze the genomic heterozygosity state in a 70-kilobase genomic region surrounding the *PRKCSH* locus (average heterozygosity between 0.4 and 0.5). Genomic regions were polymerase chain reaction amplified, followed by either restriction analysis (rs34095, rs311786, and rs311805) or sequencing (rs313624 and rs313624) (Supplementary Table 2).