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.

0016-5085/$36.00
doi:10.1053/j.gastro.2011.08.004

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).

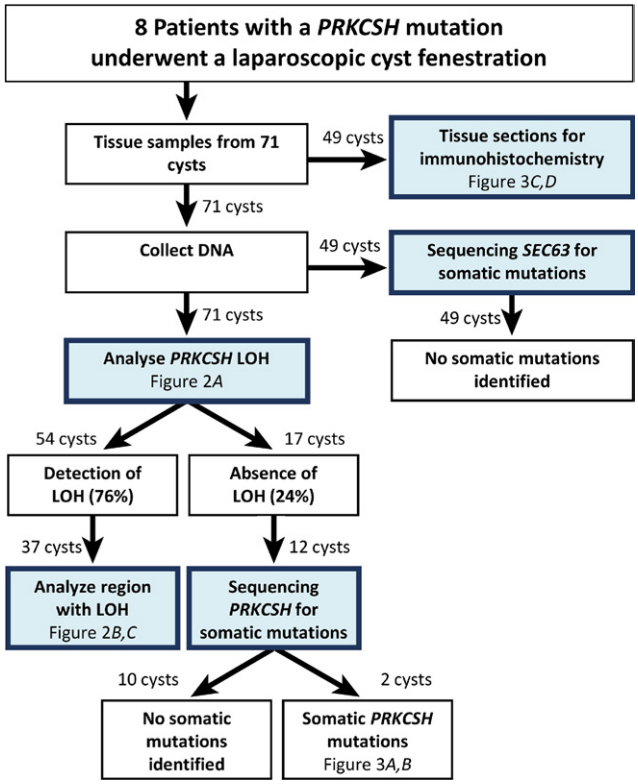


Figure 1. Somatic mutation analysis. Flow chart of sample collection and analysis of 71 cysts from 8 patients with PCLD who have a *PRKCSH* germline mutation. Cyst epithelial cells were isolated from all cysts with laser microdissection for subsequent DNA isolation and LOH analysis. In addition, samples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

Only those SNPs that were homozygous in the germline of the patient were analyzed in the DNA derived from cyst epithelium.

4. SEC63 sequencing. To identify any somatic mutations in *SEC63*, we sequenced, in all frozen tissue samples, the *SEC63* exons and flanking intronic sequences (as described for genotyping) on amplified cyst epithelial DNA.

Statistical Analysis

To calculate the 95% confidence interval of the proportion of cysts with LOH, we used the Newcombe–Wilson method for proportions without continuity correction (Supplementary Figure 1).[14]

Immunohistochemistry

Tissue sections (7 μm) for immunohistochemistry were obtained together with tissue sections for laser microdissection from the same cysts. Frozen sections were mounted on SuperFrost Plus glass slides (Thermo Scientific, Waltham, MA), allowed to dry at room temperature, and stored at −80°C or used directly. First, slides were washed in phosphate-buffered saline (PBS) and fixed in 4% formaldehyde/phosphate buffer for 30 minutes at room temperature. Endogenous peroxidase was blocked in 0.15% H_2O_2/Tris-buffered saline for 10 minutes, followed by Tris-buffered saline wash. Next, samples were microwave heated and boiled for 10 minutes in sodium citrate buffer, pH 6, cooled down to room temperature, and washed in PBS. Endogenous avidin/biotin was blocked (Vector Laboratories, Burlingame, CA), followed by overnight incubation with 1:200 mouse anti-hepatocystin antibody (sc10774; Santa Cruz Biotechnology, Santa Cruz, CA) in 1% bovine serum albumin/PBS. After washing, tissue sections were incubated for 1 hour with secondary antibody (biotinylated anti-mouse immunoglobulin G; Vector Laboratories), washed, and bound to horseradish peroxidase using the ABC method (Vector Laboratories). Detection was performed with the use of diaminobenzidine tetrahydrochloride as substrate and enhanced with copper sulfate. Nuclei were counterstained with Mayer's hematoxylin.

Immunohistochemistry scoring was performed as described previously. Briefly, hepatocystin staining in cyst epithelium was compared with normal bile duct staining. Results were classified as negative (hepatocystin staining being absent from cyst epithelium) or positive (present).[10]

Results

Germline Mutations

We collected samples from 8 patients with a pathogenic *PRKCSH* mutation who also underwent laparoscopic cyst fenestration: 5 patients with a c.1341-2A>G mutation and 3 patients with a c.292+1G>C mutation. Both germline mutations affect a *PRKCSH* splice site leading to a premature stop codon.[15] All *PRKCSH* mutations were present in a heterozygous state. No *SEC63* germline mutations were detected in these patients.

Somatic LOH in Majority of PCLD Cysts

Somatic mutation analysis showed loss of the wild-type *PRKCSH* allele in 76% (54/71) of the laser-microdissected cysts (Figure 2*A*). This in contrast to the surrounding liver tissue and blood of the patients, in which both the wild-type and mutated allele were still present. The percentage of cysts with LOH was comparable between the different patients, with the 95% confidence interval for all samples (n = 71) ranging from 65% to 84% (Supplementary Figure 1). To follow up on this finding, we continued with LOH region analysis and protein expression analysis and determined the role of additional somatic mutations in *PRKCSH* and *SEC63* in frozen tissue samples (49 cysts; Figure 2*A*). The laser-microdissected cysts from formalin-fixed, paraffin-embedded tissues (22 cysts; Figure 2*A*) did not provide enough DNA of sufficient quality to perform the downstream analyses and were therefore excluded.

Genomic LOH Region Varies Between Different Cysts

We examined the genomic region surrounding the *PRKCSH* locus using 5 common SNPs encompassing a 70-kilobase region (Figure 2*B* and *C*). Only informative SNPs (heterozygous in the germline of the patient) were analyzed in the cyst DNA samples. We found that 97% (36/37) of the cysts showing LOH at the site of the germline mutation had at least one neighboring homozygous SNP. Furthermore, in 65% (24/37) of the cysts, all informative SNPs were homozygous, indicating that LOH extended beyond the 70-kilobase region. We found both homozygous and heterozygous SNPs in 38% (14/37) of the cysts, suggesting that a genomic breakpoint occurred within this region.

Somatic Substitutions in PCLD Cyst Epithelium

In the 12 cysts without LOH, we sequenced all coding exons and flanking intronic *PRKCSH* sequences and identified a single base pair substitution in 2 heterozygous cysts from patient 1 (Figure 3*A* and *B*). This finding was confirmed by several independent experiments and no somatic mutations were identified in the control samples (patient blood and nonepithelial cells dissected from the same tissue), confirming the site-specific nature of this genomic event. Both mutations lead to the change of a conserved amino acid and are expected to affect protein function according to in silico analysis with PolyPhen, SIFT, and HOPE.[16,17]

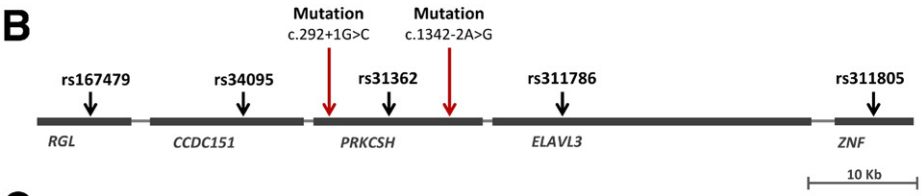
In brief, phenylalanine at position 75 is highly hydrophobic and is predicted to be buried within the core of the protein. In case of the p.F75S mutation, phenylalanine will be replaced by serine, which causes a loss of hydrophobic interactions in the core of the protein. In addition, phenylalanine and serine differ in size, which will affect the structure and stability of the protein. For the p.C500Y mutation, cysteine is replaced by tyrosine. This cysteine is highly conserved at this position among a large range of species, highlighting its importance. Further, tyrosine is bigger than cysteine and probably will not fit in the core of the protein, thereby disrupting the structure of the protein. Lastly, loss of a cysteine residue often affects a disulfide bond.

A

	Age*	Sex	Tissue samples	Heterozygous germline mutation	# of cyst analyzed	Heterozygous cysts	Cysts with LOH
Patient 1	35	F	Frozen	*PRKCSH* c.1341-2A>G	14	3 (21%)	11 (79%)
Patient 2	39	F	Frozen	*PRKCSH* c.1341-2A>G	12	3 (25%)	9 (75%)
Patient 3	42	F	Frozen	*PRKCSH* c.1341-2A>G	9	2 (22%)	7 (78%)
Patient 4	42	F	Frozen	*PRKCSH* c.1341-2A>G	9	2 (22%)	7 (78%)
Patient 5	54	F	Frozen	*PRKCSH* c.292+1G>C	5	2 (40%)	3 (60%)
Patient 6	48	F	Formalin fixed	*PRKCSH* c.292+1G>C	13	2 (15%)	11 (85%)
Patient 7	40	F	Formalin fixed	*PRKCSH* c.292+1G>C	7	3 (43%)	4 (57%)
Patient 8	36	F	Formalin fixed	*PRKCSH* c.1341-2A>G	2	0 (0%)	2 (100%)
Total					71	17 (24%)	54 (76%)

**Age at time of surgery*

B



C

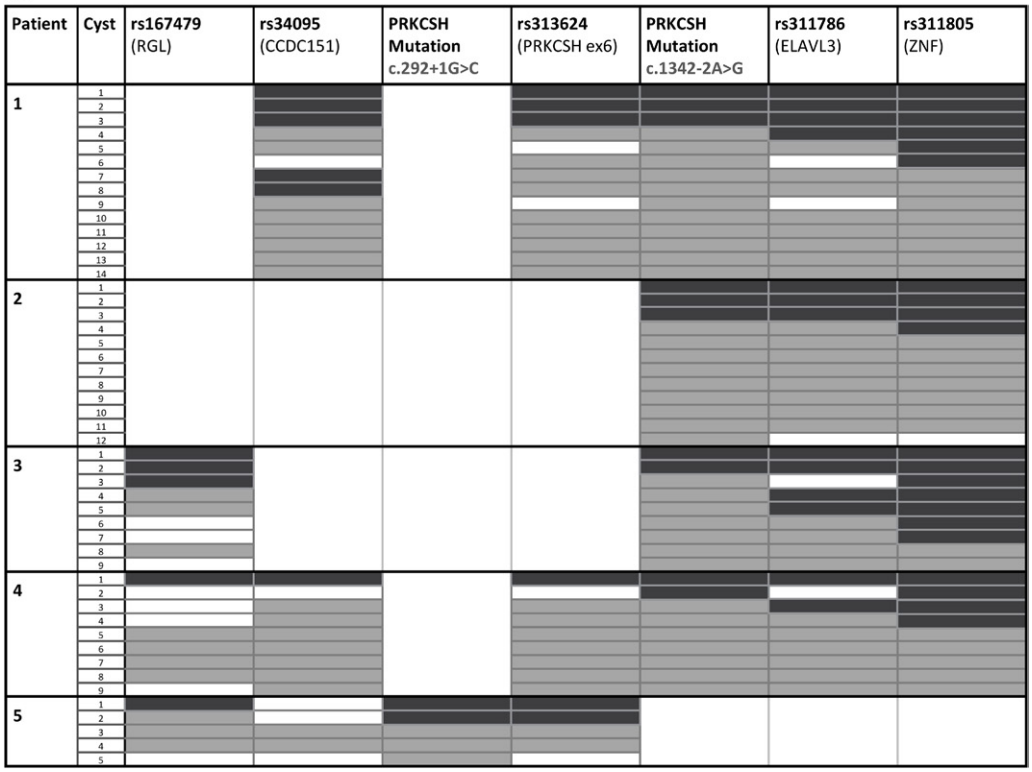


Figure 2. *PRKCSH* LOH in liver cyst epithelium. (*A*) LOH analysis of *PRKCSH* in 71 cysts from 8 different patients with PCLD who have a germline *PRKCSH* mutation. (*B*) Relative location of the 5 SNPs located in a 70-kilobase region surrounding the *PRKCSH* germline mutation used to analyze the region with LOH. (*C*) LOH region analysis in 49 cysts from frozen tissue sections. For each cyst, the heterozygosity state at the *PRKCSH* germline mutation (*c.1341-2A>G* or *c.292+1G>C*) is depicted together with the heterozygosity state of the different SNPs. Heterozygous locations are depicted in *black* and locations with LOH are depicted in *gray*. Uninformative (homozygous) SNPs and samples that continuously failed in the analysis are left blank.

Hepatocystin Expression

Hepatocystin expression was analyzed in 49 cysts using immunohistochemistry (Figure 3*C* and *D*). This showed that hepatocystin was absent from cysts with LOH, indicating that the truncated gene product from the mutated allele is not produced or not recognized by the antibody. Interestingly, hepatocystin staining was still present in cysts without somatic mutations in *PRKCSH* and in the 2 cysts with acquired point mutations (Figure 3*A* and *B*).

Trans-heterozygosity

Because germline mutations in *PRKCSH* and *SEC63* are both associated with PCLD, it is possible that somatic *SEC63* mutations contribute to cyst formation in cysts without somatic *PRKCSH* mutations. However, we

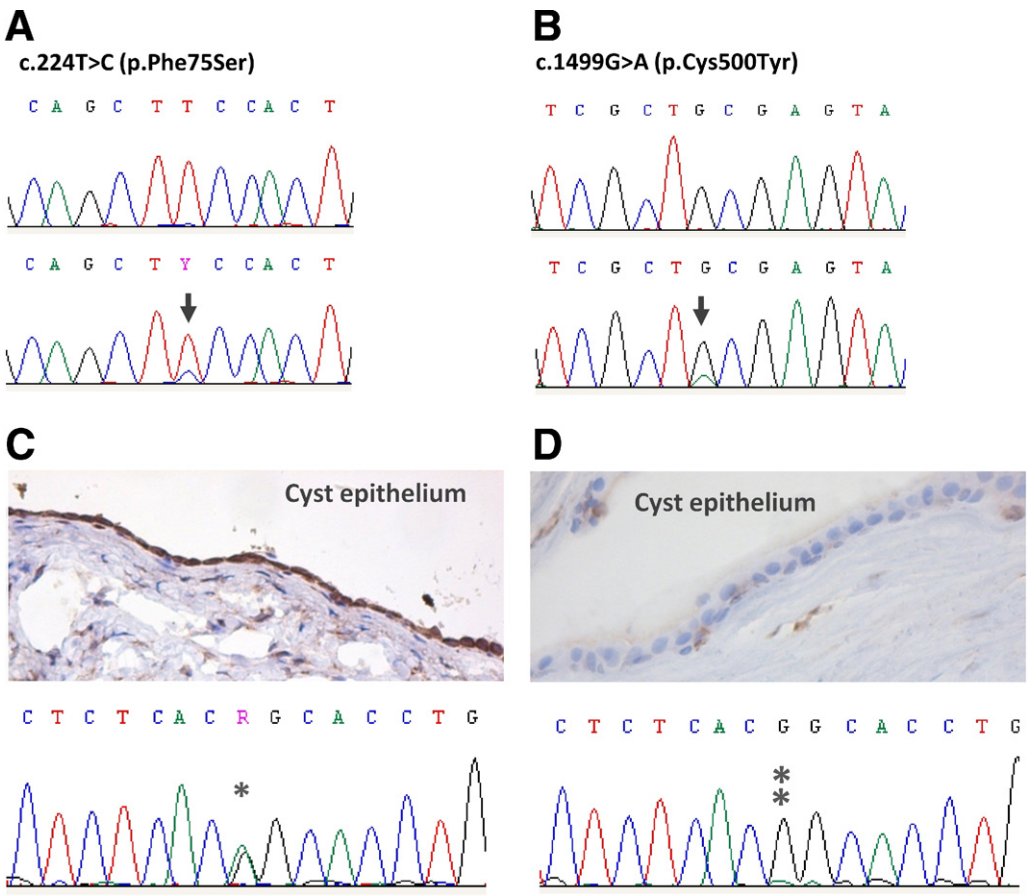


Figure 3. *PRKCSH* somatic substitutions and hepatocystin expression. (*A* and *B*) Electropherograms showing both the germline sequence (*top*) and the somatic base pair substitutions (*arrow*) identified in 2 different heterozygous cysts. (*C* and *D*) Immunohistochemical staining of hepatocystin (*brown*) and corresponding electropherogram in a genetically heterozygous cyst (*C*, *single asterisk*) or cyst with LOH (*D*, *double asterisk*) from a patient with a heterozygous c.1341-2A>G *PRKCSH* germline mutation. The immunohistochemical stainings represent a (*C*) hepatocystin-positive and (*D*) hepatocystin-negative liver cyst epithelium.

did not detect any somatic changes in the *SEC63* sequence in DNA derived from cyst epithelium compared with nonepithelial cells or DNA from whole blood from the 49 cysts analyzed. We therefore exclude trans-heterozygous *SEC63* mutations as a major cause of cyst formation in *PRKCSH* germline carriers.

Discussion

We set out to map the molecular events that occur in cyst epithelia from patients with PCLD. Our principal finding is that in patients with a heterozygous germline *PRKCSH* mutation, 76% (54/71) of the cysts lost the functional *PRKCSH* allele through LOH. The LOH region differed among cysts and extended beyond the 70-kilobase region in the majority (97%) of these cysts. We related the genomic data to immunohistochemical analysis of the tissue sections and found that cysts without LOH indeed still express hepatocystin. In addition, we sequenced the full *PRKCSH* gene in 12 cysts without LOH and found a somatic missense mutation in 2 cysts (17%; 2/12 cysts). Finally, we excluded the presence of trans-heterozygous *SEC63* mutations, the other gene associated with PCLD.

Our data support the 2-hit mechanism for disease pathogenesis for the formation of cysts in PCLD (Figure 4), which was originally described for mutations in tumor suppressor genes leading to inheritable cancers.[11] According to this model, genomic mutations naturally occur in a stochastic manner in each cell during life. For example, in familial adenomatous polyposis, somatic second-hit mutations in the *APC* gene have been associated with the formation of a polyp. Because these patients develop thousands of polyps in their intestine, the chance that a particular allele would be mutated (through LOH or other somatic mutations) was estimated to be approximately 1:500,000 per cell each year.[18]

Although the exact number may vary based on the cell type and the gene involved, somatic *PRKCSH* mutations could lead in cholangiocytes to the formation of a liver cyst stem cell. Over time, this stem cell could develop through clonal expansion and secretion into a new cyst, in which all daughter cells carry the same genomic mutation. Because the percentage of cysts with a *PRKCSH* mutation is much higher than the random mutation frequency of a gene, we believe that loss of *PRKCSH* provides a strong selective pressure for these cells and, hence, the formation of a cyst. Because most people carry 2 functional alleles of *PRKCSH*, the chance that any cholangiocyte will randomly lose both alleles from the same gene would be extremely

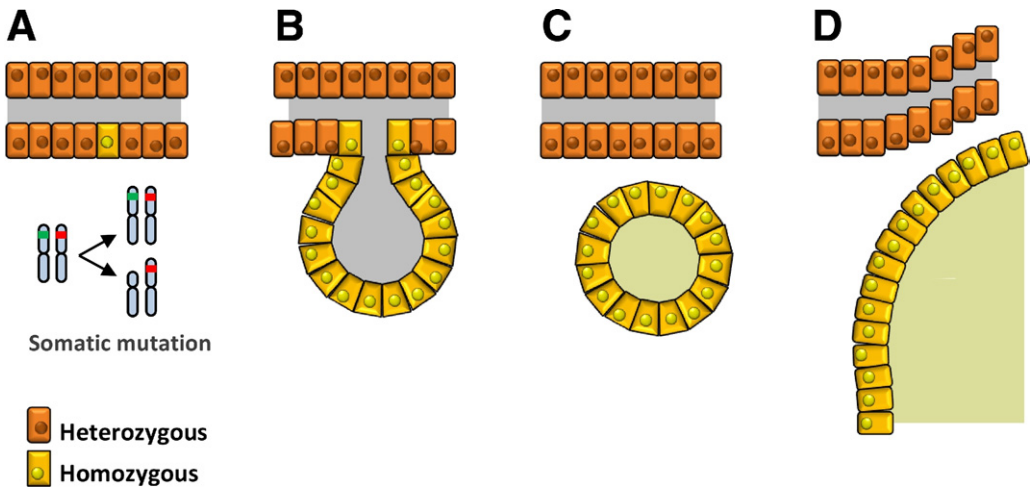


Figure 4. Model of cyst formation. (*A*) Normal cholangiocytes (in *orange*) from patients with PCLD with a *PRKCSH* mutation still carry one functional *PRKCSH* allele. However, when one of the cholangiocytes loses the functional allele through a somatic second-hit mutation, this cell can become a cyst stem cell (in *yellow*). (*B*) As this cell divides, all daughter cells will carry the same mutation and lack functional *PRKCSH*. (*C*) Although cysts may be connected to the biliary tree at first, early in their development cysts become disconnected, resulting in microscopic cysts filled with fluid. (*D*) Throughout the life of the patient, these small cysts slowly develop in the large fluid-filled cysts that are characteristic of PCLD.

small. However, losing both alleles through somatic mutations may play a role in the formation of single liver cysts in the general population.

How loss of *PRKCSH* affects cell function and how this results in the formation of a cyst is not known. Although somatic second-hit mutations could lead to loss of *PRKCSH* anywhere in the body, it is remarkable that cyst formation is only seen in the liver. This suggests that the liver-specific phenotype of PCLD is caused by a cholangiocyte-specific response to loss of *PRKCSH*. It is therefore possible that *PRKCSH* plays an important role in the growth regulation of cholangiocytes and acts as a "tumor suppressor gene." However, malignancies have never been related to patients with PCLD.

Although liver cyst epithelium displays cholangiocyte properties, the initiating somatic hit could also have occurred in a liver progenitor cell, which may explain the proliferative characteristics of cysts.[19] The genetic pattern we discovered in cysts appears to be less variable than, for example, in colorectal adenomas,[20] but we cannot exclude that polyclonality or niche succession also plays a role in the formation of polycystic livers.[21]

There are several examples of the second-hit mechanism in nonmalignant diseases, including autoimmune lymphoproliferative syndrome,[22] venous and glomuvenous malformations,[23] multi-cavernous cerebral cavernous malformations lesions,[24] and autosomal dominant polycystic kidney disease (ADPKD).[25–27]

Polycystic kidneys are the primary presentation in ADPKD, but some patients also develop polycystic livers similar to patients with PCLD.[28] ADPKD is caused by heterozygous germline mutations in *PKD1* or *PKD2*, making ADPKD and PCLD genetically and phenotypically distinct disorders. Somatic mutation analysis in kidney and liver cysts of patients with ADPKD with a *PKD1* germline mutation demonstrated LOH in 4% to 24% of kidney cysts[25,29–31] and in 10% of liver cysts.[32] Additional somatic mutations were found in 17% of kidney cysts[31] and in 28% of liver cysts.[32] Even though *PKD1* mutation analysis was hampered by technical difficulties, the proportion of kidney and liver cysts with somatic mutations was much higher compared with cysts from patients with *PRKCSH*, whereas the proportion of LOH was much lower. This also holds true for *PKD2* mutated patients, in who somatic mutations were found in 15% to 64% of kidney cysts[33–35] and 11% of liver cysts,[34] whereas LOH was present in only 0 to 12% of kidney cysts[33–35] and in none of the 9 liver cysts analyzed.[34]

One possible explanation for the difference between ADPKD and PCLD might be that the genomic region surrounding *PRKCSH* is more prone to LOH. Some regions in the DNA are known to be more susceptible to DNA double-strand breaks and misrepair. However, the region harboring *PRKCSH* is not a known hotspot for genomic rearrangements. Genomic mutations leading to LOH include deletions, translocations, or mitotic recombination,[36] but which mechanism causes LOH in PCLD cysts is not clear.

We found that the region with LOH varied greatly among the different cysts, even in those coming from the same patient. This is consistent with the idea that second-hit mutations occur randomly in the genome and supports the hypothesis that each cyst develops independently as the result of an independent somatic mutation. The presence of second hits in cysts from all patients with PCLD tested indicates that this is not a chance event limited to a single patient. The vast majority of cysts show LOH (76%), which indicates that LOH is the most likely mode of inactivation for this gene. The remaining 24% of cysts represent all the other mutations that may lead to cyst formation.

We have detected missense *PRKCSH* mutations in 2 of the 12 cysts, but we may have missed *PRKCSH* mutations in the remaining cysts. Using exon sequencing, we will not detect exon spanning deletions or translocations, thereby underestimating the proportion of cysts with somatic mutations. In addition, methylation or mutations in regulatory sequences such as the promoter region may affect gene transcription of the wild-type *PRKCSH* allele. However, immunohistochemistry showed that hepatocystin was still expressed in the heterozygous cysts, suggesting that *PRKCSH* gene expression is not silenced in these cysts.

In addition, there may be modes of cyst formation involving other genes than *PRKCSH*. The concept of trans-heterozygosity as a 2-hit mechanism has precedence in ADPKD.[31,35] In patients with a heterozygous germline mutation in *PKD1* or *PKD2*, cysts can develop due to a second hit in the remaining allele of the same gene or due to a somatic mutation in one allele of the other ADPKD gene. The best candidate for trans-heterozygosity in PCLD is *SEC63*, the other gene known to be involved in PCLD. However, we did not detect any mutations in *SEC63* and other candidates for trans-heterozygous mutations could include genes with a functional interaction to *PRKCSH* and other genes known to be involved in cytogenesis such as *PKD1*, *PKD2*, or *PKHD1*.[37,38]

The high proportion of cysts with loss of the *PRKCSH* wild-type allele suggests that somatic second-hit mutations, with consequent loss of hepatocystin, are an important step in cyst formation in PCLD. This finding is essential for our understanding of the molecular mechanism underlying cyst formation and shows that cholangiocytes are the primary affected cell type. It also indicates that PCLD is recessive at the cellular level, resulting from hepatocystin loss rather than hepatocystin haploinsufficiency. How loss of *PKRCSH* affects the cholangiocytes is not clear, but *PRKCSH* may hold some characteristics similar to tumor suppressor genes because loss of both copies of the gene is associated with cyst formation.

Supplementary Material

Note: To access the supplementary material accompanying this article, visit the online version of *Gastroenterology* at www.gastrojournal.org, and at doi: 10.1053/j.gastro.2011.08.004.

References

1. Van Keimpema L, de Koning DB, van HB, et al. Patients with isolated polycystic liver disease referred to liver centres: clinical characterization of 137 cases. Liver Int 2011;31:92–98.
2. Drenth JPH, te Morsche RH, Smink R, et al. Germline mutations in PRKCSH are associated with autosomal dominant polycystic liver disease. Nat Genet 2003;33:345–347.
3. Li A, Davila S, Furu L, et al. Mutations in PRKCSH cause isolated autosomal dominant polycystic liver disease. Am J Hum Genet 2003;72:691–703.
4. Davila S, Furu L, Gharavi AG, et al. Mutations in SEC63 cause autosomal dominant polycystic liver disease. Nat Genet 2004;36:575–577.
5. Drenth JPH, Martina J, Morsche RHMT, et al. Molecular characterization of hepatocystin, the protein that is defective in autosomal dominant polycystic liver disease. Gastroenterology 2004;126:A675.
6. Janssen MJ, Waanders E, Woudenberg J, et al. Congenital disorders of glycosylation in hepatology: the example of polycystic liver disease. J Hepatol 2010;52:432–440.
7. Desmet VJ. Congenital diseases of intrahepatic bile-ducts—variations on the theme ductal plate malformation. Hepatology 1992;16:1069–1083.
8. Everson GT, Taylor MR, Doctor RB. Polycystic disease of the liver. Hepatology 2004;40:774–782.
9. Waanders E, Venselaar H, te Morsche RH, et al. Secondary and tertiary structure modeling reveals effects of novel mutations in polycystic liver disease genes PRKCSH and SEC63. Clin Genet 2010;78:47–56.
10. Waanders E, Croes HJ, Maass CN, et al. Cysts of PRKCSH mutated polycystic liver disease patients lack hepatocystin but express Sec63p. Histochem Cell Biol 2008;129:301–310.
11. Knudson AG. Mutation and cancer: statistical study of retinoblastoma. Natl Acad Sci U S A 1971;68:820–823.
12. Van Keimpema L, Ruurda JP, Ernst MF, et al. Laparoscopic fenestration of liver cysts in polycystic liver disease results in a median volume reduction of 12.5%. J Gastrointest Surg 2008;12:477–482.
13. Van Dijk MCRF, Rombout PDM, Dijkman HBPM, et al. Improved resolution by mounting of tissue sections for laser microdissection. Mol Pathol 2003;56:240–243.
14. Newcombe RG. Two-sided confidence intervals for the single proportion: comparison of seven methods. Stat Med 1998;17:857–872.
15. Waanders E, te Morsche RH, de Man RA, et al. Extensive mutational analysis of PRKCSH and SEC63 broadens the spectrum of polycystic liver disease. Hum Mutat 2006;27:830.
16. Ng PC, Henikoff S. Predicting the effects of amino acid substitutions on protein function. Annu Rev Genomics Hum Genet 2006;7:61–80.
17. Venselaar H, Te Beek TA, Kuipers RK, et al. Protein structure analysis of mutations causing inheritable diseases. An e-Science approach with life scientist friendly interfaces. BMC Bioinformatics 2010;11:548.
18. Hornsby C, Page KM, Tomlinson I. The in vivo rate of somatic adenomatous polyposis coli mutation. Am J Pathol 2008;172:1062–1068.
19. Reya T, Morrison SJ, Clarke MF, et al. Stem cells, cancer, and cancer stem cells. Nature 2001;414:105–111.
20. Thirlwell C, Will OC, Domingo E, et al. Clonality assessment and clonal ordering of individual neoplastic crypts shows polyclonality of colorectal adenomas. Gastroenterology 2010;138:1441–1454, 1454.
21. Leedham SJ, Thliveris AT, Halberg RB, et al. Gastrointestinal stem cells and cancer: bridging the molecular gap. Stem Cell Rev 2005;1:233–241.
22. Puck JM, Straus SE. Somatic mutations—not just for cancer anymore. N Engl J Med 2004;351:1388–1390.
23. Brouillard P, Boon LM, Mulliken JB, et al. Mutations in a novel factor, glomulin, are responsible for glomuvenous malformations ("glomangiomas"). Am J Hum Genet 2002;70:866–874.
24. Akers AL, Johnson E, Steinberg GK, et al. Biallelic somatic and germline mutations in cerebral cavernous malformations (CCMs): evidence for a two-hit mechanism of CCM pathogenesis. Hum Mol Genet 2009;18:919–930.
25. Qian F, Watnick TJ, Onuchic LF, et al. The molecular basis of focal cyst formation in human autosomal dominant polycystic kidney disease type I. Cell 1996;87:979–987.
26. Qian F, Germino GG. "Mistakes happen": somatic mutation and disease. Am J Hum Genet 1997;61:1000–1005.
27. Reeders ST. Multilocus polycystic disease. Nat Genet 1992;1:235–237.

28. Drenth JP, Chrispijn M, Nagorney DM, et al. Medical and surgical treatment options for polycystic liver disease. Hepatology 2010; 52:2223–2230.
29. Brasier JL, Henske EP. Loss of the polycystic kidney disease (PKD1) region of chromosome 16p13 in renal cyst cells supports a loss-of-function model for cyst pathogenesis. J Clin Invest 1997; 99:194–199.
30. Koptides M, Constantinides R, Kyriakides G, et al. Loss of heterozygosity in polycystic kidney disease with a missense mutation in the repeated region of PKD1. Hum Genet 1998;103: 709–717.
31. Koptides M, Mean R, Demetriou K, et al. Genetic evidence for a trans-heterozygous model for cystogenesis in autosomal dominant polycystic kidney disease. Hum Mol Genet 2000;9:447–452.
32. Watnick TJ, Torres VE, Gandolph MA, et al. Somatic mutation in individual liver cysts supports a two-hit model of cystogenesis in autosomal dominant polycystic kidney disease. Mol Cell 1998;2: 247–251.
33. Koptides M, Hadjimichael C, Koupepidou P, et al. Germinal and somatic mutations in the PKD2 gene of renal cysts in autosomal dominant polycystic kidney disease. Hum Mol Genet 1999;8: 509–513.
34. Pei Y, Watnick T, He N, et al. Somatic PKD2 mutations in individual kidney and liver cysts support a "two-hit" model of cystogenesis in type 2 autosomal dominant polycystic kidney disease. J Am Soc Nephrol 1999;10:1524–1529.
35. Watnick TJ, He N, Wang KR, et al. Mutations of PKD1 in ADPKD2 cysts suggest a pathogenic effect of trans-heterozygous mutations. Nat Genet 2000;25:143–144.
36. Foulkes WD. Inherited susceptibility to common cancers. N Engl J Med 2008;359:2143–2153.
37. Fedeles SV, Tian X, Gallagher AR, et al. A genetic interaction network of five genes for human polycystic kidney and liver diseases defines polycystin-1 as the central determinant of cyst formation. Nat Genet 2011;43:639–647.
38. Harris PC, Torres VE. Polycystic kidney disease. Annu Rev Med 2009;60:321–337.

Received December 28, 2010. Accepted August 8, 2011.

Reprint requests

Address requests for reprints to: Joost P. H. Drenth, MD, PhD, Department of Gastroenterology and Hepatology, Radboud University Nijmegen Medical Centre, PO Box 9101, 6500 HB Nijmegen, The Netherlands. e-mail: joostphdrenth@cs.com; fax: (31) 24-3540103.

Acknowledgments

The authors thank Hanka Venselaar from the Center for Molecular and Biomolecular Informatics, Radboud University Nijmegen Medical Centre, for her support of the in silico analysis of somatic *PRKCSH* mutations and the Dutch pathology laboratories for collecting the samples. The laser microdissection was performed at the Microscopic Imaging Centre of the Radboud University Nijmegen Medical Centre.

Conflicts of interest

The authors disclose no conflicts.

Funding

Supported by a grant of the Institute of Genetic and Metabolic Diseases of the Radboud University Nijmegen Medical Centre. This study sponsor is not involved in study design or in collection, analysis, and interpretation of data.

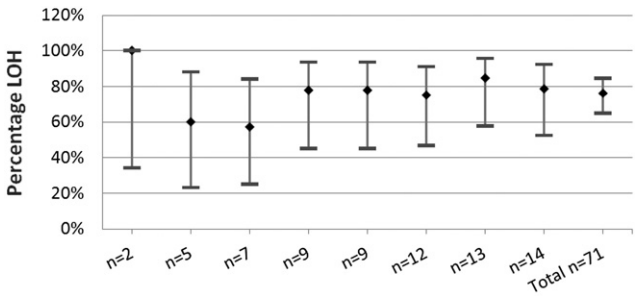
Supplementary Table 1. Primers for Sequencing

Exon	Amplicon (base pairs)	Annealing temperature (°C)	MgCl (*mmol/L*)	Primer name	Sequence
PRKCSH coding exons					
2–3	534	55	1.75	*PRKCSH Ex 2 F*	GTAGCCCTCTCCCACTGAC
				PRKSCH Ex 3 R	TGATGGATGAACAGAGAAAAGG
4–5	393	55	1.5	PRKSCH Ex 4 F	GTGGGTCAGGGGCTCTTATC
				PRKSCH Ex 5 R	GGAGCCAGGCAAAGTCTT
6	239	56	1.5	PRKSCH Ex 6 F	AGACCTGGTGGATCCTAAGT
				PRKSCH Ex 6 R	CAAGCCCACACTATGAG
7	240	64	2.0	PRKSCH Ex 7 F	TGGAGTAGAGGCAGGGAGGT
				PRKSCH Ex 7 R	GGTGAGTCCTTGGCCATTTC
8	215	59	1.75	PRKSCH Ex 8 F	GGGGTGACAGAGGTGGCTTC
				PRKSCH Ex 8 R	GGGGAACCCAAGGAGGATCT
9	197	61	1.75	PRKSCH Ex 9 F	AACAGGTGGGCCACATGGT
				PRKSCH Ex 9 R	CCCTAGAAGTCCCCAACCAAG
10	175	57	1.75	PRKSCH Ex 10 F	CGTGGTGGCCTAGATCTTGA
				PRKSCH Ex 10 R	GGCAGTGTGGTGGGAGGAC
11–12	527	63	1.75	PRKSCH Ex 11 F	GGGGCAGAGACACCGAGGCT
				PRKSCH Ex 12 R	ATCTTCCCTCGACCTGTGC
13–14	514	63	2.0	PRKSCH Ex 13 F	CCCGTTCCCCATCCTCCTG
				PRKSCH Ex 14 R	GCCTCCCACCCTGCCAGT
15–17	604	65	1.5	PRKSCH Ex 15 F	TGTGGGAGGAGGCTGGAATC
				PRKSCH Ex 17 R	CAGGCCGACGAGACTCCAC
SEC63 coding exons					
1	300	60	1.5	SEC63 Ex 1F	GGGAGTGCAGAGCGTGGTCG
				SEC63 Ex 1R	GCACCGTCCCTGTCATCCCG
2	245	56	2.0	SEC63 Ex 2F	CCTTCACTTGTGAAAAATGGTT
				SEC63 Ex 2R	CACAATGACTTATTCATCATTAC
3	280	59	2.0	SEC63 Ex 3F	TGCCTTGACCTTGCCATAAT
				SEC63 Ex 3R	AATAGACAATTTCTTTTAGAATGAGGA
4	394	50	2.0	SEC63 Ex 4F	AAAATGGATTTTGTCTTTCTAAAA
				SEC63 Ex 4R	GTATGTTGCCAGGGATGGTC
5	287	56	2.0	SEC63 Ex 5F	AATGAGTTGGTTGGCTAATGG
				SEC63 Ex 5R	GCTCAATTTATCTATGTTAAACTCCA
6	291	60	2.0	SEC63 Ex 6F	TTGTGTTGTGGGGGAGAGTT
				SEC63 Ex 6R	TGAATGAATGGCACACCAGT
7	212	60	2.0	SEC63 Ex 7F	TAGTGGCTCCCCCATTGTAA
				SEC63 Ex 7R	GGGAGACTCCAAAACATGTCA
8	303	54	1.5	SEC63 Ex 8F	TCCCAAAGTGCTGGGATTAC
				SEC63 Ex 8R	TTAAGGAATATATATCAACCCCACA
9–10	556	60	2.0	SEC63 Ex 9F	TGCATGTTTAGAAAAGTGCTTCA
				SEC63 Ex 10R	CCCAGCCTCAGGTATTTCTTT
11	284	60	2.0	SEC63 Ex 11F	TTAGTTTTGGGCCACAGTGA
				SEC63 Ex 11R	ACTGAACTGGCCGACAGAAG
12	397	60	2.5	SEC63 Ex 12F	TGTGAATTTTGTTTTTGTGATGAA
				SEC63 Ex 12R	TGTTAACAGAACCACCTGAGAGA
13	330	60	2.5	SEC63 Ex 13F	TTGCTGGATTTTGTTGTGGT
				SEC63 Ex 13R	TGTTTTGAGAATCCTGAGTCAAA
14	247	53	2.0	SEC63 Ex 14F	TTGGGTAAGCTAAGTTTTGC
				SEC63 Ex 14R	TGACTTTGACAATGAGGGAA
15–16	580	53	2.0	SEC63 Ex 15F	TGCAGTTTGAAAGATTGCTTTG
				SEC63 Ex 16R	GAAGCTGTACACGTAAGACTTGAA
17	374	58	2.0	SEC63 Ex 17F	TAGTGAAATTGTCATCGAGTCAG
				SEC63 Ex 17R	CGAGCAAGCAAACAAATGAA
18	266	53	2.0	SEC63 Ex 18F	GGAATAATACCTGGAAATCCTTAA
				SEC63 Ex 18R	CACACGACAGAGGGCTAAAA
19	290	59	2.0	SEC63 Ex 19F	GCAGTTTGTCAGTGCAGAGG
				SEC63 Ex 19R	AAAAAGATATATGAAGCAGCATGA
20	298	54	2.0	SEC63 Ex 20F	TGGTCTAGGAATTTTAGGGAGTG
				SEC63 Ex 20R	GCATGAGATGACTTCTTTTTCC
21	311	60	2.5	SEC63 Ex 21F	AAACCTTTTGTTTACCAAGATTTC
				SEC63 Ex 21R	GTTCTGTACTGTTTCTGGTTTATGA

Supplementary Table 2. LOH (Region) Analysis

Germline mutation or SNP[a]	Amplicon (base pairs)	Annealing temperature (*°C*)	MgCl (*mmol/L*)	Primer name	Sequence	Digestion[b] or sequencing
LOH analysis on formalin-fixed tissue						
292+1G>C	155	62	2.0	PRKSCH ex 4 form F	CTTCCACTGCACCAACACTG	Sequencing
				PRKSCH ex 4 form R	GCAGAAGCCAAGATCAGACC	
1341-2A>G	100	65	2.0	PRKSCH ex 16 form F	AGCCCTCAGCACCCTGTGTCTCTC	Sequencing
				PRKSCH ex 16 form R	CAGCCCGTGCCTTGCTCATACTTCA	
LOH analysis on frozen tissue						
c.292+1G>C	393	55	1.5	PRKSCH ex 4 F	GTGGGTCAGGGGCTCTTATC	Digestion with *Dde*I
				PRKSCH ex 5 R	GGAGCCAGGCAAAGTCTT	
c.1342-2A>G	604	65	1.5	PRKSCH ex 15 F	TGTGGGAGGAGGCTGGAATC	Digestion with *Ban*I
				PRKSCH ex 17 R	CAGGCCGACGAGACTCCAC	
LOH region analysis on frozen tissue						
rs311786	190	55	1.5	ELAVL3 F	CCTTTCTCTTTTGGGCACG	Digestion with *Mbo*II
				ELAVL3 R	GCAAACCCCAACACCTCTC	
rs311805	221	64	1.5	ZNF F	GCGGGCTCATCACTCAGGA	Digestion with *Msp*I
				ZNF R	GCTGGCCACCACCTCACC	
rs34095	186	64	1.5	CCDC151 F	CGCAAGGAGACTAAGGCACT	Digestion with *Sac*II
				CCDC151 R	CCACTTTCTCATCTCCCTGC	
rs167479	221	55	1.5	RGL F	ATTCTATGTCCCCCACTCTG	Sequencing
				RGL R	TCCTCCAAAAAATCTTCCAG	
rs313624	135	55	1.5	SNP PRKCSH ex6 F	CCAGGTAGAGTGTTCCAGGC	Sequencing
				SNP PRKCSH ex6 R	GATTCTCCAGCCCCATTTG	

[a]dbSNP build 129.
[b]Enzymes from New England Biolabs (Ipswich, MA).



Supplementary Figure 1. Confidence interval.