Loss of Heterozygosity Is Present in SEC63 Germline Carriers with Polycystic Liver Disease

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**Abstract**

Polycystic liver disease (PCLD) is an autosomal dominant disorder characterised by multiple fluid filled cysts in the liver. This rare disease is caused by heterozygous germline mutations in PRKCSH and SEC63. We previously found that, in patients with a PRKCSH mutation, over 76% of the cysts acquired a somatic 'second-hit' mutation in the wild type PRKCSH allele. We hypothesise that somatic second-hit mutations are a general mechanism of cyst formation in PCLD which also plays a role in PCLD patients carrying a SEC63 germline mutation. We collected cyst epithelial cells from 52 liver cysts from three different SEC63 patients using laser microdissection. DNA samples were sequenced to identify loss of heterozygosity (LOH) mutations and other somatic mutations in cyst epithelial DNA. We discovered somatic SEC63 mutations in patient 3 (1/14 cysts), but not in patient 1 and 2 (38 cysts). Upon review we found that the germline mutation of patient 1 and 2 (SEC63 c.1703_1705delAAG) was present in the same frequency in DNA samples from healthy controls, suggesting that this variant is not causative of PCLD. In conclusion, as somatic second-hit mutations also play a role in cyst formation in patients with a SEC63 germline mutation, this appears to be a general mechanism of cyst formation in PCLD.


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**Introduction**

Polycystic liver disease (PCLD; MIM# 174050) is a rare, dominantly inherited, disorder characterised by multiple fluid filled cysts in the liver. The cysts, which develop from bile duct epithelial cells (cholangiocytes), increase in size and number over time and can lead to a massive increase in liver volume [1]. So far two genes, PRKCSH (MIM# 177060) [2,3] and SEC63 (MIM# 608648) [4], have been associated with the development of PCLD. A systematic sequencing effort demonstrated that PRKCSH mutations account for 15% of the PCLD cases, whereas SEC63 mutations can be found in 6% [5].

In the case of a dominant disorder it is not always clear how the heterozygous mutations can lead to disease and which mechanisms are involved. The mutations can either lead to the production of a mutated protein which disrupts the cell function, or result in loss of functional gene product which can lead to misregulation of dosage dependent genes. Often, loss of a single allele will not have severe consequences for the function of the cell and only after the remaining allele is lost this will cause disease.

Recently, we demonstrated that in PCLD patients harbouring a heterozygous PRKCSH mutation, over 76% of the cysts acquired a somatic ‘second-hit’ mutation in PRKCSH [6]. These mutations lead to total loss of functional PRKCSH in the cyst epithelium suggesting that PCLD is recessive on a cellular level. This finding is consistent with reports on other cystic diseases, such as autosomal dominant polycystic kidney disease (ADPKD) [7–12], where somatic second-hit mutations also are present in cyst epithelia. We could also show that loss of the wild type allele corresponded to loss of PRKCSH gene product hepatocystin in these cells [6,13]. Much less is known about how the heterozygous mutations in SEC63 lead to cyst formation. On an immunohistological level, cysts from PRKCSH and SEC63 patients show a different expression pattern for various proteins including MUC1 and C-crbB-2. Furthermore, no loss of SEC63 protein has been reported in cysts form patients with a germline mutation in this gene, which could reflect a different mechanism of cyst development in patients with SEC63 germline mutation.

Although there is genetic and immunohistological heterogeneity among PCLD patients, the clinical presentation, the presence of a normal bile duct system and the focal growth of the cysts on the other hand, are features shared by all PCLD patients [15]. This suggests that the mechanism of cyst formation through second-hit mutations is similar among different genetic forms of PCLD. We therefore hypothesise that, similar to the situation in PRKCSH, somatic second-hit mutations are also an important step in cyst formation in patients with a SEC63 germline mutation.

To this end we analysed 52 cyst samples from 3 patients carrying a SEC63 mutation. In one cyst sample we found loss of heterozygosity (LOH), whereas we did not find any somatic changes in 30 samples derived from the other two patients. After reviewing the different germline mutations we found that somatic inactivation only occurred against the background of the severe truncating germline mutation.

These results show that somatic second-hit mutations play a role in cyst formation of both PRKCSH and SEC63 mutation carriers.
Materials and Methods

Ethics Statement
Liver tissue and blood samples of patients were obtained and stored in the course of treatment following the Dutch Code for the proper secondary use of human tissue. Use of this tissue for research was reviewed and approved by the regional ethics review board “Commissie Mensgebonden Onderzoek (CMO) regio Arnhem-Nijmegen”.

DNA and Tissue Samples
We used the following strategy to obtain all known liver cyst samples from SEC63 germline mutation carriers within the Netherlands. Patients were selected based on sequencing results from our molecular diagnostic laboratory, which routinely performs diagnostic tests for PCLD. Each PCLD patient is tested for PRKCSH as well as SEC63 and the database now holds 505 patients. Using this database we could readily identify 29 patients with a SEC63 mutation. We cross checked these patients against the Dutch National Pathology database to find those patients who had undergone laparoscopic cyst fenestration because of severe symptoms [16] and for whom cyst tissue samples would be available. Following this strategy we identified 3 SEC63 germline mutation carriers for whom blood and tissue samples were available.

All patients were female, and age at the time of surgery varied between 33 and 41 years-of-age. We used fresh tissue samples that had been snap-frozen immediately after excision and stored at −80°C until analysis (n = 38; 2 patients), or formalin-fixed paraffin-embedded liver tissue samples that had routinely been collected for pathological examination (n = 14; 1 patient). All samples were collected with appropriate ethics approval; written informed consent for the use of secondary tissue was obtained from all patients.

We collected whole blood from 1000 healthy controls (2000 chromosomes) which were recruited after advertisement in local papers from the same geographical region as our patients.

Genotyping
We screened patient DNA from whole blood for germline mutations in SEC63 and PRKCSH using direct sequencing as described previously [6]. In brief, DNA from whole blood was isolated using the PureGene DNA isolation kit (Genta Systems, Minneapolis, Minnesota, USA) and stored at 4°C. Exons and flanking intronic sequences were amplified using polymerase chain reaction (PCR) with specific primers. 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, USA). Names of SEC63 mutations refer to the NM_007214.4 transcript according to the HGVS guidelines.

We screened genomic DNA samples of 1000 healthy controls for the SEC63 c.1703_1705delAAG mutation using PCR with the forward primer 5′-TGTGAATTCCTAGAAGGCACCCTTG-3′ and reverse 5′-TGTCGTTTTCTATCCCACTAA-3′ and the SEC63 c.1703_1705delAAG mutation (forward 5′-TAGTGAATTCCTAGAAGGCACCCTTG-3′ and reverse 5′-CGAGCAAGCAGAAAATGAA-3′) followed by sequencing. Somatic mutations in Polycystic Liver Disease

DNA Isolation from Dissected Cells
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 two days with occasional agitation; proteinase K solution (>600 mAU/ml) was added in two steps (5 µl on day 1 and 5 µl on day 2); after digestion and addition of buffer ATL, samples were incubated at 90°C for 1 hr to promote reverse cross-linking of the DNA. To obtain enough material for the SEC63 and PRKCSH sequencing, cyst epithelial DNA was amplified using a commercially available whole genome amplification kit (GenomePlex WGA, Sigma-Aldrich, Saint Louis, MO, USA) and purified (GenElute PCR Clean-Up Kit, Sigma-Aldrich) prior to analysis. Sample amplification was performed in duplicate to control for any mutations resulting from the whole genome amplification procedure.

Somatic Mutation Analysis
We conducted the following analyses on DNA isolated from laser dissected samples:

(1) SEC63 LOH analysis. The region of the germline mutation was amplified using specific primers for the SEC63 c.958G>T mutation (forward 5′-TGGGAGTCTAGACGAGGCGAT-3′ and reverse 5′-TGGGAGTCTAGACGAGGCGAT-3′) and the reverse primer 5′-CGAGCAAGCAGAAAATGAA-3′ followed by sequencing to determine the heterozygosity state of the germline mutation in both cyst epithelia and control liver cell samples.

(2) SEC63 and PRKCSH sequencing. All SEC63 and PRKCSH coding exons and flanking intronic sequences were sequenced (as described for genotyping) on amplified DNA to detect somatic mutations in the cysts. DNA isolated from whole blood of the patient was used as reference sample.

LOH Region Analysis
We used 5 heterozygous SNPs across chromosome 6 to analyse the heterozygosity state in cyst DNA using PCR with specific primers: rs2012025 (forward 5′-GGGACAGCAGAAAAATCCTTTG-3′, reverse 5′-CTACGTCCTATATATACAGCC-3′), rs13220047 (forward 5′-TGGGAGTCTAGACGAGGCGAT-3′ and reverse 5′-GGGAGTCTAGACGAGGCGAT-3′), rs675117 (forward 5′-AGCCCAACAAGGATTTGGAATG-3′, reverse 5′-TTTAAATGCACTCACCAGAATTG-3′), rs12210583 (forward 5′-GGGAGTCTAGACGAGGCGAT-3′, reverse 5′-GGGAGTCTAGACGAGGCGAT-3′), rs10946279 (forward 5′-GGGAGTCTAGACGAGGCGAT-3′, reverse 5′-GGGAGTCTAGACGAGGCGAT-3′), followed by sequencing.
Immunohistochemistry

Tissue sections (4 μm) from formalin-fixed paraffin-embedded
PCLD liver tissue were obtained together with tissue sections for
laser micro dissection. Sections were mounted on SuperFrost
Plus glass slides (Thermo Scientific Waltham, MA, USA) using
a water bath and allowed to dry overnight at 37°C. Prior to the
staining the tissue sections were deparaffinized with xylene and
hydrated with alcohol and distilled water. Samples were
microwave heated and boiled for 10 min in Na-Citrate buffer
pH 6, cooled down to room temperature, washed in phosphate
buffered saline (PBS) and incubated for 30 min with 1% BSA/
PBS (Bovine Serum Albumin, Sigma-Aldrich). Endogenous
Avidin/Biotin was blocked (Vector laboratories, Burlingame,
CA, USA) followed by overnight incubation with 1:100 rabbit
anti-SEC63 antibody (kind gift from Prof. Dr. Enno Hartmann,
University of Lübeck, Germany), 1:200 mouse anti-hepatocystin
antibody (sc10774, Santa Cruz, CA, USA) or 1:400 mouse anti-
Cytokeratin 19 (MU246-UC, BioGenex, San Ramon, CA) in
1% BSA/PBS. For each staining we included a negative control
in which the primary antibody was omitted. Endogenous
peroxidase was blocked in 0,3% H2O2/PBS for 10 min after
1%BSA/PBS. For each staining we included a negative control
for 1 hr with secondary antibody (biotinylated anti-mouse or
anti-rabbit IgG, Vector laboratories), washed and bound to
Horse-Radish-Peroxidase using the ABC method (Vector
laboratories). Detection was carried out with the use of
diaminobenzidine tetrahydrochloride (DAB) as substrate and
laboratories). Detection was carried out with the use of
amino acids.

Somatic Mutation Analysis

Using laser microdissection we collected epithelial cells from 52
cysts and found LOH to be present in one cyst from patient #3
(Table 1, Figure 1B). Next, we screened the coding sequence of
SEC63 and PRKCSH by Sanger sequencing in the remaining cysts
of patient #1 and patient #2, no somatic mutations were present.
Due to the formalin fixation in the samples of patient #3 we could
not sequence SEC63 and PRKCSH to detect other somatic
mutations in these samples.

LOH Region Analysis

We used 5 informative (heterozygous) single nucleotide
polymorphisms (SNPs) across chromosome 6 to determine the extent
of the region with LOH (Figure 1A, B and C). This demonstrated
that the LOH region extends over 6 kb from the site of the
germine mutation SEC63 c.958G>T (in exon10) to exon 6 in the
same gene. The SNP (rs13220047) located at a distance of 82 kb
remains heterozygous indicating that a genomic breakpoint
occurred within this region. In tumour samples LOH regions
are often telomeric [19,20] and extent from one breakpoint
towards the end of the chromosome. This was not the case in our
sample, where the telomeric SNPs remained heterozygous
(Figure 1C).

Immunohistochemistry

Immunostaining of SEC63 and hepatocystin showed that the
hepatocystin staining was similar between cysts, whereas for
SEC63 the intensity of the staining was reduced in the cyst with
LOH but appears normal in the cysts without loss of heterozy-
gosity (Figure 1D).

Somatic Mutations Only Present in Patient with Severe
Germline Mutation

We found LOH in one of the 14 cysts of patient #3, while
somatic mutations were conspicuously absent from samples from
the other 2 patients (38 cysts). We reviewed the different
underlying SEC63 germline mutations and found that the germline
mutation of patient #3 is potentially much more severe than those
of the other 2 patients. Both patient #1 and #2 carry a heterozygous
SEC63 c.1703_1705delAAG mutation which will
lead to an in-frame deletion of a single amino acid: glutamate at
position 568 in the protein. In contrast, the germline mutation of
patient #3 (SEC63 c.958G>T) results in a premature stop codon
after 319 amino acids (p.Glu320X), which deletes 441 out of 760
amino acids.

To determine whether the SEC63 c.1703_1705delAAG mutation
is a true pathogenic mutation or represents a rare (benign)
polymorphism, we determined the frequency of this mutation in
the normal population by high resolution melting analysis of this
region in genomic DNA of 1000 healthy subjects. We found this
specific amino acid deletion in 6 out of 2000 normal chromosomes

Table 1. Somatic mutation analysis.

<table>
<thead>
<tr>
<th>Age*</th>
<th>Sex</th>
<th>Tissue samples</th>
<th>Heterozygous germline mutation</th>
<th># of cysts analyzed</th>
<th>Cysts with LOH</th>
</tr>
</thead>
<tbody>
<tr>
<td>38</td>
<td>F</td>
<td>Frozen</td>
<td>SEC63 c.1703_1705delAAG</td>
<td>34</td>
<td>0 (0%)</td>
</tr>
<tr>
<td>41</td>
<td>F</td>
<td>Frozen</td>
<td>SEC63 c.1703_1705delAAG</td>
<td>4</td>
<td>0 (0%)</td>
</tr>
<tr>
<td>33</td>
<td>F</td>
<td>Formalin fixed</td>
<td>SEC63 c.958G&gt;T</td>
<td>14</td>
<td>1 (7%)</td>
</tr>
</tbody>
</table>

*Age at time of surgery.

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(0.30%), which is similar to the frequency present in a sample of 373 PCLD patients (5/746 chromosomes, 0.67%) as depicted in Figure 2. The difference between both groups was not statistical different (p = 0.18). This suggest that the genomic variant SEC63 c.1703_1705delAAG is not a pathogenic mutation causing PCLD which may explain the absence of any somatic mutations in this gene.

**Discussion**

Our data show that somatic second-hit mutations do occur in SEC63 mutation carriers with PCLD, which is in line with the second-hit model for disease pathogenesis.

We did not detect somatic mutations in two out of three patients and we hypothesised this was related to the nature of the germline mutation. After reviewing the germline mutations we found that
the allele frequency of the SEC63 c.1703_1705delAAG mutation did not differ between our PCLD patients and the healthy population. This indicates this variant is a (rare) polymorphism which is not associated with PCLD. The literature reports this mutation several times in relation to PCLD [4,5] but segregation in a family with PCLD has never been shown. Davila et al. reported that they did not find this variant in 360 healthy chromosomes [4], which can be explained by the low allele frequency of this variant. We therefore believe that patients 1 and 2 do not have a pathogenic SEC63 mutation, but belong to the cohort of PCLD patients in which the genetic cause is still unknown. This is important to know as currently patients with PCLD are being screened, classified and advised based on wrong information.

As PRKCSH and SEC63 are ubiquitously expressed in the human body, it remains unclear why germline mutations would lead to a liver specific disorder. The gene product of SEC63, SEC63, is thought to play a role in protein transport across the endoplasmic reticulum (ER) membrane, whereas PRKCSH codes for hepatocystin and is part of a heterodimer complex involved in folding of glycoproteins in the ER [21,22]. However, targeted inactivation of both PRKCSH and SEC63 in an ADPKD mouse model leads to a synergistic increase in disease severity, which suggests that these genes share a biological pathway [23].

Although the frequency of somatic second-hit mutations varies between different genes and genetic disorders, the difference in somatic mutations between cysts from PRKCSH patients and this SEC63 patient is remarkable. We found LOH in only 7% (1/14) of SEC63 mutated cysts, whereas in PRKCSH germline carriers the majority of cysts (76%) acquired LOH [6]. Although the numbers are low, this difference is statistically significant (p<0.00001) and could indicate that these two genes have a different susceptibility to somatic LOH. The immunohistochemical data confirmed loss of SEC63 protein in the cysts with LOH, which indicates that the truncated gene transcript from the mutated SEC63 c.958G>T allele may no longer be translated into protein or is not recognised by the antibody.

Our study was restricted by the limited amount of available patient tissue. However, we identified and collected all known PCLD liver tissue samples that had been stored within the Netherlands. We obtained samples from three patients, but only one patient carried a bona fide pathogenic SEC63 germline mutation. Furthermore, the available tissue was formalin fixed which was not a problem for the laser microdissection procedure, but did reduce the yield of the DNA isolation. We were able to get a clear read on the LOH status of all samples, but the sequence efficiency was affected in material derived from formalin-fixed paraffin-embedded tissue. Therefore there may still be presence of, yet unidentified, somatic mutations in these samples.

There is a body of evidence emanating from recent studies supporting the concept of somatic mutations as part of the genetic pathogenesis of benign and malignant disorders. It was recently shown that hamartomata that are part of the Proteus syndrome arise from somatic activating mutations in oncogene AKT1 [24]. In patients with acute lymphoblastic leukemia and cervical cancer somatic mutations play an important role in the development and prognosis of the disease [25,26]. Lastly, recurrent somatically acquired mutations of the SF3B1 gene can be demonstrated in subtypes of myelodysplastic syndromes in which ring sideroblasts are a prominent feature [27]. Collectively, these data demonstrate that genetic inactivation through somatically acquired mutations help to understand the tissue specificity of certain malignant but also non-malignant disorders.

In conclusion, we have now shown that in both PRKCSH and SEC63 somatic second-hit mutations can occur which supports the notion that somatic second-hit mutations are part of the genetic mechanism in cyst formation in PCLD.

Acknowledgments

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The allele frequency of the SEC63 c.1703_1705delAAG mutation is shown in Figure 2. The table below provides the allele frequency and confidence interval of the SEC63 c.1703_1705delAAG gene variant in genomic DNA from PCLD patients and healthy controls.

<table>
<thead>
<tr>
<th></th>
<th># Chromosomes analysed</th>
<th># SEC63 delAAG</th>
<th>Allele frequency</th>
<th>95% Confidence interval</th>
</tr>
</thead>
<tbody>
<tr>
<td>Healthy subjects</td>
<td>2000</td>
<td>6</td>
<td>0.30%</td>
<td>Lower limit (2.5%)</td>
</tr>
<tr>
<td>PCLD patients</td>
<td>746</td>
<td>5</td>
<td>0.67%</td>
<td>Upper limit (97.5%)</td>
</tr>
</tbody>
</table>

**Figure 2. Allele frequency of SEC63 c.1703_1705delAAG.** Allele frequency and confidence interval of the SEC63 c.1703_1705delAAG gene variant in genomic DNA from PCLD patients and healthy controls. doi:10.1371/journal.pone.0050324.g002
Author Contributions

Conceived and designed the experiments: MJJ JS RHMM JPHD. Performed the experiments: MJJ JS RHMM. Analyzed the data: MJJ JS RHMM JPHD. Contributed reagents/materials/analysis tools: JPHD. Wrote the paper: MJJ JPHD. Revised the article critically for important intellectual content: MJJ JS RHMM JPHD.

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