Somatic *PKD2* Mutations in Individual Kidney and Liver Cysts Support a "Two-Hit" Model of Cystogenesis in Type 2 Autosomal Dominant Polycystic Kidney Disease

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Abstract. An intriguing feature of autosomal dominant polycystic kidney disease (ADPKD) is the focal and sporadic formation of renal and extrarenal cysts. Recent documentation of somatic *PKD1* mutations in cystic epithelia of patients with germ-line *PKD1* mutations suggests a "two-hit" model for cystogenesis in type 1 ADPKD. This study tests whether the same mechanism for cystogenesis might also occur in type 2 ADPKD. Genomic DNA was obtained from 54 kidney and liver cysts from three patients with known germ-line *PKD2* mutations, using procedures that minimize contamination of cells from noncystic tissue. Using intragenic and microsatellite markers, these cyst samples were screened for loss of heterozygosity. The same samples were also screened for somatic mutations in five of the 15 exons in *PKD2* by single-stranded conformational polymorphism analysis. Loss of heterozygosity was found in five cysts, and unique intragenic mutations were found in seven other cysts. In 11 of these 12 cysts, it was also determined that the somatic mutation occurred nonrandomly in the copy of *PKD2* inherited from the unaffected parent. These findings support the "two-hit" model as a unified mechanism for cystogenesis in ADPKD. In this model, the requirement of a somatic mutation as the rate-limiting step for individual cyst formation has potential therapeutic implications.

Autosomal dominant polycystic kidney disease (ADPKD) is a common genetic disorder that affects approximately 1 in 1000 live births and is an important cause of chronic renal disease (1,2). Two disease genes, which account for most cases of ADPKD, have been recently identified (3–5). Germ-line *PKD1* mutations account for at least 80% of all clinical cases, while germ-line *PKD2* mutations account for most of the remainder (1,6). Clinically, the spectrum of renal and extrarenal manifestations of both type 1 and type 2 ADPKD overlaps completely (1). Renal cyst formation is a universal feature, while other manifestations may include extrarenal cysts, cardiac valvular abnormalities, colonic diverticulae, and cerebral aneurysms (1). An intriguing feature of this disease is the focal nature of renal and extrarenal cyst formation, which increases in an age-dependent manner (7). Additionally, significant variability in intrafamilial disease severity has been well documented (7,8). These observations suggest that additional factor(s) other than the germ-line mutation are required for the phenotypic variability in ADPKD (7). Indeed, in patients with germ-line

PKD1 mutations, recent studies have shown that most of the cysts examined are clonal in origin (9,10). Moreover, both loss of heterozygosity and intragenic *PKD1* mutations have also been documented in cystic epithelia from these patients (9–11). In all cases where it could be determined, the somatic mutation occurred in the copy of *PKD1* inherited from the unaffected parent (9–11). These findings suggest a "two-hit" model for type 1 ADPKD in which cystogenesis requires the inactivation of both copies of *PKD1* (9–11). In the current study, we tested whether the same mechanism for cystogenesis might also occur in type 2 ADPKD.

Materials and Methods

Patients and Study Samples

We have previously characterized the germ-line *PKD2* mutations in all three study patients. For patient UT1270, we found a splice site mutation (*i.e.*, IVS5+1G→A) predicted to result in aberrant splicing of exon 5 (unpublished data). The consequence of this mutation at the mRNA level has not been characterized. In contrast, the mutations in patients JHU496 (*i.e.*, C1390T in exon 6; R464X) and UT1500 (*i.e.*, 2125InsA in exon 11; frameshift 720–724X) are both predicted to be protein truncating (12). Each of these mutations segregated only in the affected members of the family and was not found in at least 100 normal chromosomes. Genomic DNA was isolated from blood leukocytes of the study patients using a published protocol (13). Thirty-two cysts were isolated from both kidneys of patient UT1270 at autopsy, nine cysts were isolated from a nephrectomized kidney of patient JHU496, and 13 cysts were isolated from the liver of patient

UT1500 at the time of liver transplantation. All experimental procedures used in this study were previously approved by the Institutional Review Board of the University of Toronto, Toronto; Johns Hopkins University, Baltimore; and the Health Sciences Center of St. Johns, Newfoundland.

Preparation of Cyst DNA

Two published procedures that minimize contaminating cells from noncystic tissue were used to isolate the epithelial cells from each cyst (9,10). The contents of each cyst from patients UT1270 and JHU496 were drained using a needle and syringe, and the needle was left in the cyst. The cyst cavity was rinsed at least three times with Ca^{2+}- and Mg^{2+}-free phosphate-buffered saline (PBS), followed by injection and incubation with ethylenediaminetetra-acetic acid-containing PBS for 20 min (9). The cyst contents were then collected and the DNA was extracted using a commercial kit (Puregene, Gentra System, Minneapolis, MN) or a spin column (Qiagen, Chatsworth, CA) (9). The domes of single cysts from patient UT1500 were carefully dissected from surrounding tissue, removed, and rinsed in PBS. The interior surface of the dome of these cysts was then scraped with a clean razor blade, leaving the cyst wall intact. DNA was extracted from cells separated from the cyst wall by scraping using a spin column (Qiagen) (10).

Loss of Heterozygosity Analysis

For this analysis, we exploited the presence of sequence variants associated with the heterozygous germ-line mutation of each study patient. PCR of genomic DNA from blood leukocytes and individual cyst samples of the study patient was performed to amplify the germ-line mutation, using previously published primers and conditions (12,14). The PCR products from each sample were then purified using a spin column (Qiagen) and subjected to single-stranded conformational polymorphism (SSCP) analysis (see PKD2 Intragenic Mutation Screening). Loss of heterozygosity was defined as at least a 50% reduction in the intensity of the wild-type band compared with the mutant conformer. In the cysts that exhibited loss of heterozygosity with these intragenic markers, we also examined a second marker, D4S1563, for independent confirmation of these findings. This microsatellite marker is approximately 0.5 cM from the 3' end of PKD2 and was shown previously to exhibit a heterozygous pattern in all three study patients (5,15). The genotyping was performed by ^{32}P α-dCTP labeling of the PCR products and analyzed after polyacrylamide gel electrophoresis and autoradiography (15). Two criteria were used to determine the presence of loss of heterozygosity. (1) Both marker alleles amplified from blood leukocyte DNA were equal in intensity. (2) There was at least a 50% reduction in the intensity of one of the marker alleles amplified from the cyst DNA. For each of these methods, the PCR was repeated at least twice for any sample that exhibited loss of heterozygosity.

PKD2 Intragenic Mutation Screening

We screened individual cyst samples for somatic PKD2 mutations in exons 2, 3, 5, 6, and 11. At least 50% of all of the germ-line mutations have been reported to occur in these exons (12). Using SSCP, we screened all 54 cyst samples for each of the five exons that were PCR-amplified, using previously published primers and conditions (12,14). PCR products from the leukocyte DNA of the study patients were included as controls. Each PCR fragment was screened for a minimum of four different temperatures (i.e., 4, 12, 18, and 28°C), using the thermoflow electrophoresis temperature control (ETC) system (Novex, San Diego, CA) (12). All PCR products were separated by electrophoresis on either a 4 to 20% or a 20% polyacrylamide gel and visualized by silver staining (12). All SSCP variants detected were confirmed by repeated PCR and SSCP. Both strands of the PCR fragments containing the SSCP variants were then sequenced directly using the fluorescence dideoxy terminator method and analyzed using an ABI 373 DNA sequencer (Applied Biosystems, Foster City, CA).

Parental Origin of the Copy of PKD2 Containing the Somatic Mutation

Three methods were used to determine the parental origin of the copy of PKD2 containing the somatic mutation in a cyst. First, for the cysts that exhibited a loss of the wild-type SSCP conformer (i.e., C9, C23, C26, and C30 in patient UT1270 and C8 in patient JHU496), we sequenced the PCR products directly. Reduction to homozygosity of the heterozygous germ-line mutation was interpreted as due to deletion of the copy of PKD2 inherited from the unaffected parent. Second, we genotyped all of the available family members of each study patient using D4S1563 to determine the cosegregation of specific marker allele with the disease. Using this information, we inferred the parental origin of the copy of PKD2 with the somatic deletion. Third, in six of the seven cysts in which we found small PKD2 intragenic mutations, we amplified and subcloned genomic PCR products containing both the germ-line and somatic mutations. For cysts C5, C8, and C11 from patient UT1270, we amplified and subcloned an approximately 4-kb genomic fragment containing both exons 5 and 6 using published primers (i.e., IF1c and IR8), the Advantage II enzyme mix (Clontech, Palo Alto, CA), and an annealing temperature of 60°C (14). For the remaining cysts (i.e., C14 from patient UT1270, and cysts C1 and C3 from patient JHU496), we amplified and subcloned a genomic fragment containing either exon 5 or 6. At least three independent clones from each of these cysts were sequenced at both sites of the germ-line and somatic mutations.

Results

Somatic PDK2 Mutations in Individual Cystic Epithelia

We found loss of heterozygosity at the PKD2 locus in five of 54 (9%) cysts (Table 1). In all of these cysts, a differential loss of the normal SSCP conformer was evident. Figure 1a shows the SSCP pattern of the exon 5 PCR products from blood leukocyte DNA of a healthy subject and cyst DNA samples from patient UT1270. An extra upper band is seen in all of the cyst samples and cosegregates with the germ-line mutation. Examples of loss of heterozygosity are shown by the reduced intensity of the lower band in cysts C9 and C30. Similarly, Figure 1b shows the SSCP pattern of the exon 6 PCR products from blood leukocyte DNA of a healthy subject and cyst DNA samples from patient JHU496. An extra middle band is seen in all of the cyst samples and cosegregates with the germ-line mutation. Loss of heterozygosity is shown by the loss of the lower band in cyst C8.

We also found small intragenic mutations in seven of 54 (13%) cysts after screening five of 15 exons in PKD2. Most of these mutations are predicted to result in a truncated protein product (Table 1). For cyst C8 of patient UT1270, we found a 3-bp in-frame deletion (i.e., 1434del3) that did not appear to activate a cryptic splice site. The functional significance of this mutation is unknown since it is predicted to delete only a single amino acid, isoleucine, within the second transmembrane do-

Table 1. PKD2 somatic mutations in individual kidney and liver cysts[a]

Patient	Cyst	Mutation[b]	Location	Predicted Effect[c]
UT1270 from TOR-PKD31 (germ-line mutation: IVS5+1G → A; aberrant splicing of exon 5)				
right kidney	C5	1365del13	Exon 6	Frameshift 455 → 456X
	C8	1434del3	Exon 6	del479I
	C9	LOH		Loss of wild-type allele
	C11	1450delA	Exon 6	Frameshift 484 → 513X
	C14	1220del34	Exon 5	Frameshift 407 → 440X
left kidney	C23	LOH		Loss of wild-type allele
	C26	LOH		Loss of wild-type allele
	C30	LOH		Loss of wild-type allele
JHU496 from NFL-PKD16 (germ-line mutation: C1390T; R464X in exon 6)				
kidney	C1	1510del27	Exon 6	del a.a.504-512
	C3	1505InsT	Exon 6	Frameshift 502 → 525X
	C8	LOH		Loss of wild-type allele
UT1500 from TOR-PKD8 (germ-line mutation: 2152InsA; frameshift 720 → 724X in exon 11)				
liver	C7	IVS2-8del19	Exon 3	Aberrant splicing of exon 3

[a] Ins, insertion; del, deletion; LOH, loss of heterozygosity; A, adenosine; C, cytidine; G, guanosine; T, thymidine; I, isoleucine; X, stop codon.
[b] Nucleotide numbering starts with the first in-frame ATG codon of the mRNA sequence of PKD2.
[c] Codon numbering starts with the first in-frame methionine of polycystin 2.

main of polycystin 2 (5,14). For cyst C1 of patient JHU496, we found a 27-bp in-frame deletion (*i.e.*, 1510del27). This mutation is predicted to delete nine amino acids at the junction of the first intracellular loop and third transmembrane domain of polycystin 2 and may result in misfolding of the encoded protein (16).

Parental Origin of the Copy of PKD2 Containing the Somatic Mutation

In the five cysts that we found loss of heterozygosity, direct sequencing of the PCR products revealed a reduction to homozygosity of the heterozygous germ-line mutation (Figure 1, c and d). Additionally, we also found loss of the marker allele of *D4S1563* from the nondiseased parent in these same cysts (data not shown). In seven other cysts with small *PKD2* intragenic mutations, we amplified and subcloned genomic PCR fragments containing both sites of the germ-line and somatic mutations in six of them. Sequencing both sites of the germ-line and somatic mutations in individual clones localized the somatic mutations in these cysts to the copy of *PKD2* inherited from the unaffected parent (Figures 2 and 3). We were unable to assess the parental origin of the copy of *PKD2* containing the somatic mutation in cyst C7 of patient UT1500.

Discussion

In this preliminary report, we found inactivating somatic mutations in approximately 20% of 54 cysts after screening for five of 15 exons in *PKD2*. In 11 of 12 cysts in which it could be determined, the somatic mutations all occurred in the copy of *PKD2* inherited from the unaffected parent. The probability for a chance occurrence of this nonrandom segregation pattern is less than 1 in 2000 (*i.e.*, $(1/2)^{11}$) and strongly suggests that these somatic mutations are causally related to the cystogenic

process. Our data are also consistent with previous findings in type 1 ADPKD (9–11). Furthermore, recent studies of *Pkd1* and *Pkd2* "knockout" mouse models both suggest a cellular recessive mechanism for cyst formation in ADPKD (17,18). Taken together, these studies suggest a "two-hit" model for ADPKD in which germ-line and somatic inactivation of both copies of a polycystic kidney disease gene confers growth advantages for an individual cell to clonally expand into a cyst (9–11,17–19). Accordingly, the germ-line mutation is necessary but insufficient and the somatic mutation is the rate-limiting step for cyst formation (9–11,19).

Two currently unresolved controversies concerning the "two-hit" model relate to the low rates of somatic *PKD1* hits reported, as well as recent findings on polycystin expression in cystic epithelia of patients with type 1 ADPKD (9–11,20–22). The first controversy is that loss of heterozygosity was observed in only 17 to 24% of the cysts examined in patients with type 1 ADPKD (9,10,23). However, loss of heterozygosity reflects only deletion of large chromosomal regions and not small intragenic changes. Indeed, a more recent study has documented small pathologic mutations within *PKD1* in approximately 30% of the cysts examined from patients with type 1 ADPKD (11). Given the large size and complexity of *PKD1* and the limitations on the sensitivity of the mutation screening methods currently available, it is highly unlikely that somatic *PKD1* changes can be detected in every cyst from patients with type 1 ADPKD. The second controversy is that strong immunoreactivity was found in most of the cystic epithelia examined from patients with type 1 ADPKD (20–22). Since most germ-line *PKD1* mutations reported are inactivating, a high percentage of cysts with reduced or absent polycystin-1 expression are expected (23). These findings have led one group to question whether somatic loss of the wild-type *PKD1* allele is required
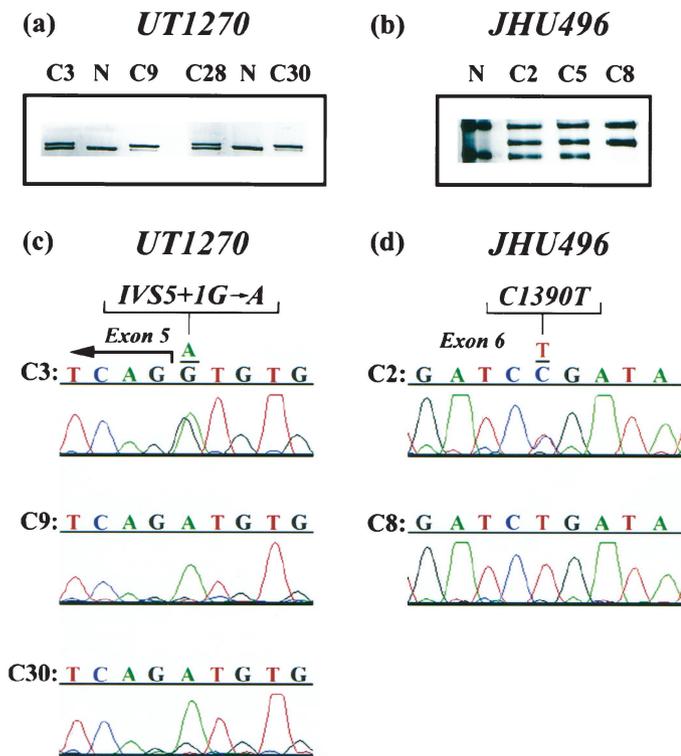
Figure 1. Loss of heterozygosity at the *PKD2* locus in cystic epithelia. (a) Single-stranded conformational polymorphism (SSCP) pattern of exon 5 PCR products from blood leukocyte DNA of a normal subject (N) and cyst DNA from patient UT1270. An extra upper band is seen in all cyst samples and cosegregates with the germ-line mutation. Examples of loss of heterozygosity are shown by the reduced intensity of the lower band in cysts C9 and C30. (b) SSCP pattern of exon 6 PCR products from blood leukocyte DNA of a normal subject (N) and cyst DNA samples from patient JHU496. An extra middle band is seen in all cyst samples and cosegregates with the germ-line mutation. Loss of heterozygosity is shown by the loss of the lower band in cyst C8. (c) Sequence tracings of exon 5 PCR products from cyst C3 in patient UT1270 reveals a heterozygous germ-line mutation (IVS5+1G→A). In contrast, reduction to homozygosity of the germ-line mutation is seen in cysts C9 and C30. (d) Sequence tracings of exon 6 PCR products from cyst C2 in patient JHU496 reveals a heterozygous germ-line mutation (C1390T). In contrast, reduction to homozygosity of the germ-line mutation is seen in cyst C8.

Figure 2. Sequence tracings of two genomic clones containing the sites of germ-line and somatic mutations in cyst C5 from patient UT1270. The sequence tracing of genomic clone #1 reveals the presence of the germ-line mutation in exon 5 (*i.e.*, IVS5+1G→A) and normal sequence in exon 6. The sequence tracing of genomic clone #2 showed a normal sequence in exon 5 and the somatic mutation in exon 6 (*i.e.*, 1365Del13).

for cystogenesis (23). However, there are several potential explanations for this discrepancy. First, if the germ-line or somatic *PKD1* mutation did not disrupt the mRNA reading frame (*i.e.*, in-frame deletions/insertions, or missense mutations), the presence of immunoreactivity in a cyst might merely reflect the detection of a nonfunctional protein. Second, it is possible that certain inactivating *PKD1* mutations may prolong the half-life of polycystin-1, as has been demonstrated for the p53 tumor suppressor gene (10,24). Third, it is also possible that *PKD1* expression is negatively regulated by polycystin-1 and that the absence of this feedback in cells without functional polycystin-1 leads to increased levels of the mutant protein. This mechanism has been proposed to explain the increased mRNA levels associated with biallelic inactivation of the patched gene in basal cell carcinomas (25). Finally, a "trans-
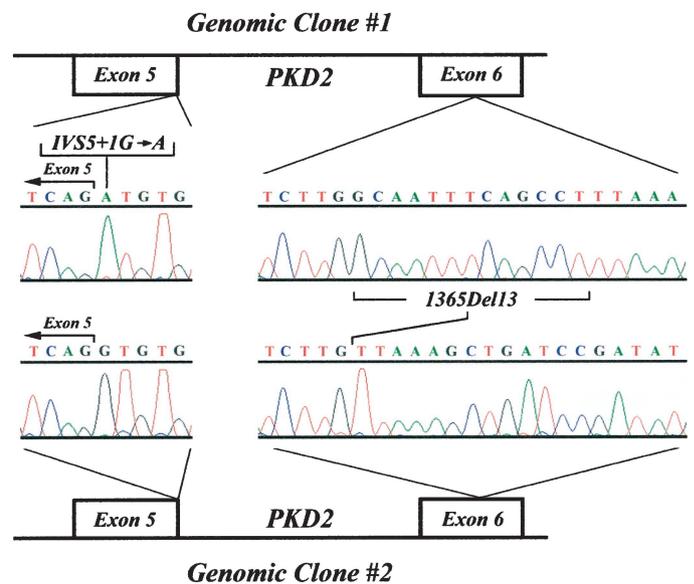
heterozygous" model has been recently proposed as an additional mechanism for cystogenesis in ADPKD (11,19). In this model, a cell with compounded heterozygous mutations of two different ADPKD genes may clonally expand into a cyst (11,19). All of these mechanisms need not be mutually exclusive from each other to account for the polycystin-1 immunoreactivity observed.

Because up to thousands of cysts may be found in a polycystic kidney with advanced disease, the "two-hit" model (including the "trans-heterozygous" variant) for ADPKD suggests a high rate of background somatic mutations in normal renal epithelial tissue (7,19). Indeed, using a functional assay for identifying inactivating mutations of the X-linked hypoxanthine phosphoribosyl transferase gene, a recent study has documented a high frequency of somatic mutations in normal human renal epithelial tissue *in vivo* (26). Moreover, these somatic events increase exponentially with age (26). However, the effects of most of these somatic mutations in individual cells are likely masked by the remaining copy of the functional gene. In contrast, a somatic *PKD* mutation in ADPKD will result in the cyst phenotype since a germ-line *PKD* mutation already exists in the same cell. A corollary of this model is that two independent somatic *PKD* mutations within single cells, which are much rarer events, may lead to sporadic renal or liver cysts in the general population.

In conclusion, our data, together with previous findings in type 1 ADPKD (9–11), support the "two-hit" model as a unified mechanism for cystogenesis in ADPKD. The extent that this model may operate to account for all of the cysts formed in a polycystic kidney needs to be assessed in future
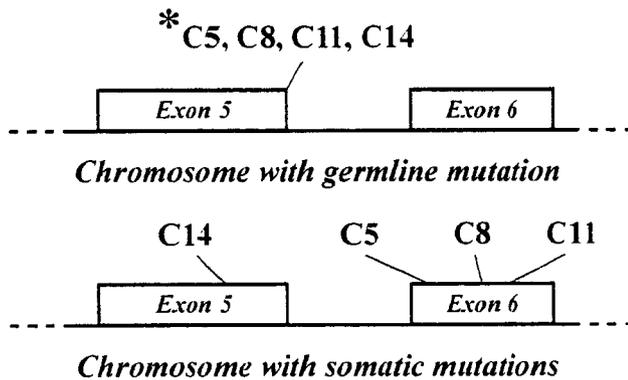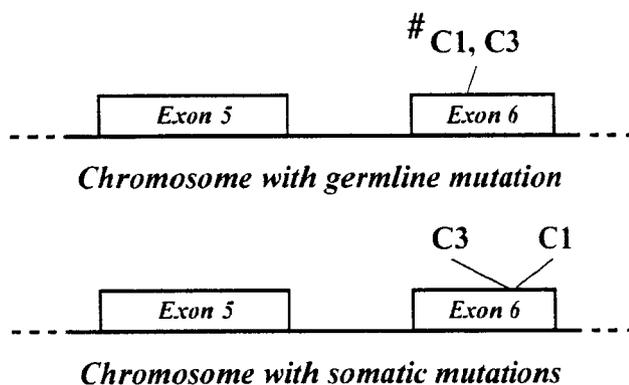
(a) UT1270:

(b) JHU496:

Figure 3. Somatic mutations in cystic epithelia occurred in the copy of *PKD2* inherited from the unaffected parent. Summary of the sequencing results of genomic clones containing the sites of both germ-line and somatic *PKD2* mutations in cystic epithelia. The location of each germ-line and somatic mutation in the two parental chromosomes for cysts C5, C8, C11, and C14 in patient UT1270 and for cysts C1 and C3 in patient JHU496 is shown schematically in panels a and b, respectively. The location of the germ-line mutation for patients UT1270 (*i.e.*, IVS5+1G→A) and JHU496 (*i.e.*, 1365Del13) is denoted by the symbols * and #, respectively. A mutually exclusive segregation pattern between the germ-line and somatic mutations in the two parental chromosomes is seen in each of these cysts. The specific germ-line and somatic mutations for these cysts are summarized in Table 1.

studies. Because it is technically much more difficult to screen the entire *PKD1* for mutations, this assessment can be more feasibly conducted by the complete screening of *PKD2* for somatic mutations in cystic epithelia of patients with type 2 ADPKD. Additionally, the "trans-heterozygous" model can also be tested as an additional mechanism for cystogenesis in ADPKD by screening for somatic *PKD1* mutations in cystic epithelia from patients with type 2 ADPKD, or *vice versa.* Under both of these models, the somatic mutation is the rate-limiting step for individual cyst formation. The factors that govern the rates of somatic hits of a polycystic kidney disease gene remain to be defined, but will likely involve a combina-

tion of genetic and environmental determinants. The identification of these factors may have therapeutic potential in ADPKD.

Addendum: A recent study has also documented somatic *PKD2* mutations in renal cystic epithelia of a patient with type 2 ADPKD (Koptides M, Hadjimichael C, Koupepidou P, Pierides A, Constantinou Deltas C: Germinal and somatic mutations in the *PKD2* gene of renal cysts in autosomal dominant polycystic kidney disease. *Hum Mol Genet* 8: 509–513, 1999). In this study, small intragenic somatic mutations, but not loss of heterozygosity, were found in nine (43%) of 21 cysts after the entire *PKD2* was screened. Of interest, the somatic mutation (*i.e.*, 197–203insC in exon 1) in seven of these nine cysts was identical. If confirmed, the latter findings would suggest a "hot-spot" for somatic *PKD2* mutations.

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