Germinal and somatic mutations in the PKD2 gene of renal cysts in autosomal dominant polycystic kidney disease

Michael Koptides1, Christos Hadjimichael1, Panayiota Koupepidou1, Alkis Pierides2 and C. Constantinou Deltas1,*

1The Cyprus Institute of Neurology and Genetics, Department of Molecular Genetics and 2Department of Nephrology, Nicosia General Hospital, Nicosia, Cyprus

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Autosomal dominant polycystic kidney disease (ADPKD) is caused by mutations in one of three genes: PKD1 on chromosome 16 accounts for ∼85% of cases whereas PKD2 on chromosome 4 accounts for ∼15%. Mutations in the PKD3 gene are rare. All patients present with similar clinical phenotypes, and the cardinal symptom is the formation of fluid-filled cysts in the kidneys. Previous work has provided data supporting the notion that cysts in ADPKD1 are focal in nature and form after loss of function of polycystin 1. This became evident by demonstrating that the normal PKD1 allele was inactivated somatically by loss of heterozygosity or by mutagenesis in a subset of renal or liver cysts examined. We show in this report, for the first time, multiple novel somatic mutations within the PKD2 gene of epithelial cells, in both kidneys of an ADPKD2 patient. From a total of 21 cysts examined, seven (33%) had the same C insertion within the inherited wild-type allele. In two other cysts, a nonsense mutation and a splice site AG deletion had occurred in a PKD2 allele that could not be identified as the inherited wild-type or mutant. We suggest that the autosomal dominant form of ADPKD2 occurs by a cellular recessive mechanism, supporting a two-hit model for cyst formation.

RESULTS AND DISCUSSION

The main feature of ADPKD pathology is the formation and development of cysts in many organs, primarily the kidneys and secondarily the liver and pancreas. However, only a small fraction of renal cells become cystic. Initially, the proposed hypothesis stated that a second hit may be needed after fetus conception, which would trigger cyst formation (13). We investigated this hypothesis by obtaining both kidneys post-mortem of an ADPKD2 patient belonging to a family where the pathogenic germinal mutation is the insertion of a cytosine residue after Leu231 in exon 2 (693insC; nucleotide number 1 is the A of the first methionine codon) (14). We isolated DNA from epithelial cells of the inner wall of well-separated individual cysts and

*To whom correspondence should be addressed. Tel: +357 2 392655; Fax: +357 2 358237; Email: deltasco@mdrtc.cing.ac.cy
cysts, three from the left kidney and four from the right (Fig. 2).

In some cases, it is not known in which allele this deletion occurred. For this, the entire coding region of the gene was examined by single strand conformational polymorphism (SSCP) and part of the gene by heteroduplex analysis. Heteroduplex analysis failed to identify any abnormal patterns, except for finding the known mutation in exon 2 (14). However, screening by SSCP revealed aberrant electrophoretic migration of PCR-amplified products in three different exons.

A new molecular species detected by SSCP around exon 4 from cyst C7 DNA was sequenced directly from PCR products. Sequencing in both directions revealed the presence of a stop codon that substituted arginine 327 (R327X) (Fig. 1A). The nucleotide substitution was a transversion of adenosine to thymidine at the first position of the codon (AGA→TGA). It was impossible to determine whether this potentially deleterious mutation had occurred in the inherited mutant or the wild-type allele. A second SSCP variant around exon 10 from cyst C19 DNA could not be elucidated by direct sequencing of PCR products. Thus, subsequent cloning of these fragments and sequencing of multiple clones revealed a de novo deletion of the conserved AG dinucleotide at the 3′ splice site in intron 9 (Fig. 1B). This mutation, denoted as 2020-2delAG, is expected to result in aberrant splicing and perhaps deletion of exon 10 or inclusion of intron 9, if no cryptic splice sites are activated. As for the previous mutation, it is not known in which allele this deletion had occurred.

Another more startling finding was within exon 1 of seven cysts, three from the left kidney and four from the right (Fig. 2). All seven cysts shared the same SSCP variant, and direct sequencing of PCR products showed the insertion of a new cytosine within a series of six consecutive cytosines (nucleotides 197–203), encoding amino acids 66–68, denoted as 197–203insC. It is impossible to determine exactly where the insertion of the cytosine occurred. However, it is expected to create a translation frameshift which would lead to incorporation of 22 novel amino acids before it reaches a stop codon. Fortuitously, we identified a nearby polymorphism at position 83, which was occupied by either G or C, encoding either arginine or proline (R28P). This polymorphism enabled us to verify that the disease was co-inherited with allele C (Fig. 3). Cloning and sequencing of cystic DNA showed that the C insertion at position 197–203 had occurred in the inherited wild-type allele, thereby rendering cystic cells devoid of normal polycystin 2 (Fig. 2).

The recurrent occurrence of this last mutation, 197–203insC, in one-third of the cysts examined, suggests that this position may represent a mutational hotspot. The insertion took place within the only sequence of six consecutive cytosines of the PKD2 coding region, something that is known to be particularly prone to mispairing due to strand slippage. In addition, the entire exon 1 sequence is 77.5% GC rich, something that may also render it prone to mutations. The finding of two variants in such a small part of exon 1, namely the insertion 197–203insC and the polymorphism R28P, may be indicative of high mutagenicity, a finding also established for the PKD1 gene. In fact, the increased mutagenicity may partly explain the high incidence of cyst formation in both ADPKD1 and ADPKD2 kidneys, and the formation of simple cysts during ageing (1). Also, polymorphism R28P, with two very dissimilar amino acids occupying the same position near the N-terminal end of polycystin 2, perhaps suggests a less important role for this part of the protein.

It is not known whether the somatic mutations preceded the development of cysts, or followed their hyperproliferation (15,16). Perhaps there is still a need for a more direct demonstration of a cause-and-effect relationship between somatic mutations and cyst formation. Nevertheless, in the light of recent experiments (12), our findings support the two-hit hypothesis for cyst formation in ADPKD2. Wu et al. had shown that the development of polycystic kidney disease in a murine model was the result of intragenic homologous recombination that led to somatic inactivation of both PKD2 genes, thereby suggesting that ADPKD2 occurs by a cellular recessive mechanism (12). If the de novo insertion of the C in seven of the cysts examined in this work had happened after cyst formation, and was not causative of cystogenesis, one would expect it to have happened with equal frequency on both alleles. However, this was not the case, since in all seven cysts the C insertion was found only in the germinal wild-type copy allele.
Figure 2. Sequencing of clones around the regions of interest showing the cystic somatic mutation 197–203insC, and the nearby polymorphism, R28P, which permitted the discrimination of alleles. Forward and reverse sequencing in the presence of dITP as a dGTP analogue and inclusion of 40% formamide within the denaturing sequencing gels were necessary for resolving misleading compressions and artefacts, caused by the high GC content of the region. (A) Forward sequencing of DNA from cyst C6 (alleles 1 and 2) and of DNA isolated from the patient’s leukocytes (allele 3), showing the region around polymorphic nucleotide 83. (B) Reverse sequencing of the same samples as in (A), showing the region around the somatic mutation. The newly introduced G in the antisense strand (allele 1) was inserted somewhere between the shown arrows. In (A) and (B), allele 1 on the left represents that arising from the somatic de novo insertion of a C between residues 197 and 203, and which occurred on the inherited wild-type allele with G at position 83. Allele 2 represents the inherited mutant with a C at position 83, and allele 3 represents the inherited wild-type allele with G at position 83. (C) Schematic representation of the three PKD2 alleles found in the DNA isolated from the patient’s leukocytes and/or from epithelial cells of the renal cysts. The polymorphic nucleotides at position 83 are underlined. Also underlined is the newly introduced C in the allele with a germinal mutation after amino acid 231 (nucleotide 693). In leukocytes, alleles 2 and 3 are present, whereas in the DNA of seven cysts, alleles 1 and 2 are present. aa, amino acid; nt, nucleotide.

Somatic mutagenesis as the triggering event of cyst formation and disease development would account for the great intra- and interfamilial phenotypic variation which clearly characterizes ADPKD (1). It would also account for the age-dependent penetrance, since the somatic mutations would happen as stochastic events triggered by endogenous or environmental factors any time during intra- and extrauterine life.

It did not escape our attention that only nine of 21 (43%) of the cysts examined showed somatic mutations. This may be due to various reasons, the most probable of which pertains to the inherent weakness of the SSCP approach we used for mutation detection. SSCP does not identify 100% of single nucleotide variants, whereas small or larger deletions around the PCR primer recognition sequence would lead to hemizygosity. Also, potential mutations in the rest of the intronic sequences would be missed since we only focused on the exonic regions and the exon–intron boundaries.

In conclusion, we have presented for the first time direct evidence supporting the two-hit hypothesis, according to which inactivation of both PKD2 genes in renal epithelial cells. One mutant gene is inherited and the second gene is inactivated through somatic second hits represented by de novo mutations in certain hotspots or elsewhere in the gene. This is certainly a finding that follows the Knudson’s two-hit model for carcinogenesis (17), and provides molecular support for the previous characterization of fluid-filled cysts as ‘neoplasia in disguise’ (18).

MATERIALS AND METHODS

Patient information

The patient belongs to a family, CY1602, that was investigated previously by DNA linkage analysis (19) and had shown clear evidence for linkage to the PKD2 locus on chromosome 4. The pathogenic mutation subsequently was shown to be an insertion of C after leucine 231 in exon two (693insC) (14). At the age of 54 years, the patient had a mild cerebrovascular accident, and at 64 years he presented with hypertension. He reached end-stage renal disease and commenced haemodialysis at the age of 70
years. He died at age 79 years, at which time both kidneys were removed on informed consent by the relatives.

Isolation of cyst epithelial cells and genomic DNA extraction

In both kidneys there were hundreds of cysts of variable size. The isolation of cystic epithelial cells and DNA extraction were performed as described previously for ADPKD1 kidneys (9,10), exercising caution to avoid non-cystic cells. This involved rinsing the inner surface of cystic slices in excess phosphate-buffered saline (PBS) and scraping it with a surgical blade, whilst keeping the cyst outer wall intact. Epithelial cells were obtained from 21 individual well-separated cysts, eight from the left and 13 from the right kidney.

Search for LOH

LOH around the PKD1 and PKD2 loci on chromosomes 16 and 4, respectively, was investigated with the use of intragenic and flanking markers as described previously (10). Since the inherited mutation in the patient was known, 693insC in exon 2, LOH was investigated easily by PCR amplification and heteroduplex analysis as previously shown (14). The co-amplification of the normal and the mutant allele gives a unique pattern by heteroduplex analysis. The proximal flanking marker D4S231 was also used for this purpose. For investigation of LOH in and around PKD1, the intragenic markers KG8 and IVS42 were used, as well as flanking markers HBAAP1, SM6 and 16AC2.5. Unfortunately, KG8 and IVS42 were not informative.

SSCP, heteroduplex analysis and DNA sequencing

The entire PKD2 gene, which is encoded in 15 exons, was screened for mutations using an exon-by-exon approach. Fourteen pairs of primers located in the flanking sequence of exons 2–15 and three pairs covering the first exon were used for mutation screening, using DNA from 21 cysts as templates, as described previously (14). The entire collection of primers used is described elsewhere (20). Exons showing aberrant SSCP patterns were either sequenced directly using the PCR sequencing kit from USB (Amersham, Cleveland, OH) according to the manufacturer’s instructions, or were sequenced after cloning into the pPCR-Script SK(+)- vector (Stratagene, La Jolla, CA). Recombinant clones represented by white colonies were easily screened for the two polymorphic alleles exploiting a restriction enzyme recognition site around the polymorphic nucleotide 83. The C allele creates a recognition site for Bsp1286I.

For all PCR amplifications, extreme precaution was exercised to avoid carry over or external DNA contaminations. Preparation of samples was within a specially used self-contained compartment (Template Tamer; Coy, MI), and blank samples containing everything but DNA were included in all series of amplifications.

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