The Molecular Basis of Focal Cyst Formation in Human Autosomal Dominant Polycystic Kidney Disease Type I

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Summary

Autosomal dominant polycystic kidney disease (ADPKD) is a common disease and an important cause of renal failure. It is characterized by considerable intrafamilial phenotypic variation and focal cyst formation. To elucidate the molecular basis for these observations, we have developed a novel method for isolating renal cystic epithelia from single cysts and have used it to show that individual renal cysts in ADPKD are monoclonal. Loss of heterozygosity was discovered within a subset of cysts for two closely linked polymorphic markers located within the PKD1 gene. Genetic analysis revealed that it was the normal haplotype that was lost. This study provides a molecular explanation for the focal nature of cyst formation and a probable mechanism whereby mutations cause disease. The high rate at which "second hits" must occur to account for the large number of cysts observed suggests that unique structural features of the PKD1 gene may be responsible for its mutability.

Introduction

Autosomal dominant polycystic kidney disease (ADPKD) is one of the most common inherited diseases of humans; it is estimated to affect 1 in 1000 of the population (Gabow, 1993). Renal cysts are the major clinical features of the disease and appear to increase in size and number throughout the lifetime of an individual. This process results in renal failure in approximately half of affected individuals by age 50 years. Although the renal lesion is the most prominent feature, ADPKD is a systemic disorder with a variety of other manifestations including liver cysts, cerebral aneurysms, and a variety of cardiac valvular abnormalities.

ADPKD exhibits considerable variability with respect to both its renal and extrarenal manifestations. Genetic heterogeneity is likely to account for at least some of these differences. Linkage studies have determined that there are at least three forms of ADPKD. PKD1, which is most common and accounts for 85%–95% of all cases, maps to chromosome 16p13.3 (Reeders et al., 1985). The second type, PKD2, which affects most of the remaining families, maps to chromosome 4q13–23 (Kimberling et al., 1993; Peters et al., 1993). A small number of families have another form, which has not yet been mapped (Bogdanova et al., 1995; Daoust et al., 1995; de Almeida et al., 1995). Although all types of ADPKD present with an identical profile of extrarenal manifestations (including liver cysts and aneurysms), PKD1 is the most severe, with a lower median survival and a higher risk of progressing to end-stage renal disease (Ravine et al., 1992).

While unique mutations in disease-causing genes may account for interfamilial variation, they cannot explain why related individuals who presumably share a common mutation have different clinical presentations. Severe childhood cases of ADPKD born into families that exhibit the classic adult presentation of the disease are dramatic examples of this phenomenon (Blyth and Ockenden, 1971; Kaariainen, 1987; Fick et al., 1993; Zerres et al., 1993). Fick et al. (1994) argued that these families are examples of anticipation and proposed that an unstable trinucleotide repeat might be responsible for these findings.

Since these initial clinical observations were made, the genes for PKD1 and PKD2 have been identified. PKD1 encodes a 14 kb mRNA that is derived from 46 exons that extend over ~50 kb of genomic DNA (The European Polycystic Kidney Disease Consortium, 1994; The American PKD1 Consortium, 1995; Hughes et al., 1995). The predicted protein of 968 amino acids has significant homology to the voltage-activated Ca\(^{2+}\) channel \(\alpha_{1C}\) as well as to a portion of polycystin. It is thought to encode an integral membrane protein whose activity is regulated by polycystin. Neither gene has any trinucleotide repeats within its mRNA that are >5 units in length, nor are any found within the complete gene sequence of PKD1.

Mutation analysis of PKD1 has been greatly hindered by high sequence similarity to its replicated loci. Nonetheless, the mutations reported to date for both PKD1 and PKD2 have been stable nucleotide substitutions, deletions, or insertions and do not explain why family members with the same germline mutation exhibit dramatic phenotypic variability (Peral et al., 1995, 1996a, 1996b; Mochizuki et al., 1996). In fact, Peral et al. (1996b) recently reported a set of fraternal twins with the same germline nonsense mutation in PKD1 yet with remarkably different phenotypes. One child had a severe infantile-onset form of the disease, whereas the other had no cysts at the age of 5 years. The authors suggested that the difference in clinical presentation was most likely due to the effect of a small number of genetic modifying factors.

Although breeding experiments in mice with recessive...
cystic disease support a role for the effect of other loci in disease expression, genetic background probably does not explain all aspects of phenotypic variability in human ADPKD (Iakoubova et al., 1995). Even within a single kidney, fewer than 1% of nephrons actually develop cysts, suggesting that cystogenesis is a focal process. Histopathologic studies of ADPKD kidneys have confirmed that cyst formation begins with the localized outgrowth of a tubule in any nephron segment, including segments with different embryologic origins (Evan and McAteer, 1992; Schäfer et al., 1994). These observations suggest that cyst formation is a two-step process and that an inherited mutation at one of the ADPKD gene loci is necessary but insufficient for cystogenesis.

We have hypothesized that the focal nature of cyst formation in ADPKD probably holds clues to an understanding of the pathogenesis of this disorder. To study this process, we have developed a novel method for isolating epithelial cells from single renal cysts that minimizes contamination by other cells and have used it to show that renal cysts in ADPKD are monoclonal. We have demonstrated loss of heterozygosity (LOH) within individual cysts for two closely linked polymorphic markers located within the PKD1 gene. Genetic analysis has confirmed that it is the normal haplotype that is lost in these specimens (samples 9 and 10). The results of three other samples that were prepared from individual cysts of donor JHU93 (Figure 1A), two failed to yield sufficient product for analysis (samples 9 and 10). The results of the clonality assay of cysts isolated from two individuals with ADPKD are presented in Figure 1 as representative examples. Of the 11 samples that were prepared from individual cysts of donor JHU93, two failed to yield sufficient product for analysis (samples 9 and 10). The results of the clonality assay of cysts isolated from two individuals with ADPKD are presented in Figure 1 as representative examples. Of the 11 samples that were prepared from individual cysts of donor JHU93, two failed to yield sufficient product for analysis (samples 9 and 10). The results of the clonality assay of cysts isolated from two individuals with ADPKD are presented in Figure 1 as representative examples. Of the 11 samples that were prepared from individual cysts of donor JHU93, two failed to yield sufficient product for analysis (samples 9 and 10). The results of the clonality assay of cysts isolated from two individuals with ADPKD are presented in Figure 1 as representative examples. Of the 11 samples that were prepared from individual cysts of donor JHU93, two failed to yield sufficient product for analysis (samples 9 and 10). The results of the clonality assay of cysts isolated from two individuals with ADPKD are presented in Figure 1 as representative examples. Of the 11 samples that were prepared from individual cysts of donor JHU93, two failed to yield sufficient product for analysis (samples 9 and 10). The results of the clonality assay of cysts isolated from two individuals with ADPKD are presented in Figure 1 as representative examples. Of the 11 samples that were prepared from individual cysts of donor JHU93, two failed to yield sufficient product for analysis (samples 9 and 10). The results of the clonality assay of cysts isolated from two individuals with ADPKD are presented in Figure 1 as representative examples. Of the 11 samples that were prepared from individual cysts of donor JHU93, two failed to yield sufficient product for analysis (samples 9 and 10). The results of the clonality assay of cysts isolated from two individuals with ADPKD are presented in Figure 1 as representative examples. Of the 11 samples that were prepared from individual cysts of donor JHU93, two failed to yield sufficient product for analysis (samples 9 and 10). The results of the clonality assay of cysts isolated from two individuals with ADPKD are presented in Figure 1 as representative examples. Of the 11 samples that were prepared from individual cysts of donor JHU93, two failed to yield sufficient product for analysis (samples 9 and 10). The results of the clonality assay of cysts isolated from two individuals with ADPKD are presented in Figure 1 as representative examples. Of the 11 samples that were prepared from individual cysts of donor JHU93, two failed to yield sufficient product for analysis (samples 9 and 10).
were deemed uninterpretable because of incomplete digestion (samples 1, 3, and 7). The remainder (samples 2, 4, 5, 6, 8, and 11) yielded a single product after digestion. Samples prepared from 16 individual cysts of donor JHU273 were similarly analyzed (Figure 1B). The clonal status of three samples could not be determined because their final rinse solutions also yielded PCR products (C3 and C15) or because no PCR product was detected (C5). Of the remaining 13 cysts, 11 (85%) yielded a single PCR product after HhaI digestion. Only 2 of the 13 cysts (C6 and C14) yielded two products, and in each instance the bands were of unequal intensity, suggesting a clonal bias.

A total of 76 renal cysts derived from 8 affected females were analyzed for clonality, and 62 cysts (82%) were found to be monoclonal (Table 1). It is very likely that the actual percentage of monoclonal cysts is >82%. In some samples, rinse solutions were not tested for luminal contamination (Table 1). Later analyses revealed that the rinse solution of approximately one half of the cysts apparently yielded PCR products, suggesting probable contamination (Figure 1). The small number of cysts that are truly polyclonal may have resulted from the fusion of two or more formerly neighboring monoclonal cysts.

**Allelic Loss of a PKD1 Intragenic Microsatellite, KG8**

It is possible that cysts may develop from a cluster of cells sharing a common X-chromosome inactivation status rather than from clonal expansion of a single progenitor cell. To prove that renal cysts are truly monoclonal and to investigate the molecular basis of clonality, we tested for LOH for PKD1 in individual cysts using the microsatellite KG8 (Snarey et al., 1994). This marker lies within the 3‘ untranslated region (3‘UTR) of the PKD1 mRNA (Figure 2). Two alleles were equally amplified in most samples. In a subset, however, only a single band was amplified. We included primers for the androgen receptor as an internal control. Two bands corresponding to the androgen receptor alleles were equally amplified in all samples, including those that had a single band for KG8. Figure 3A is a representative example of data using these markers. Two cysts from this kidney (C8 and C14) were found to have LOH. A total of 46 cysts from four donors were evaluated with KG8, and 17% were found to be hemizygous for this marker (Table 1). At least two cyst preparations per donor had loss of a KG8 allele, and in each case it was the same allele.

**Loss of a Second Closely Linked Marker, EJ1**

We sought to confirm the KG8 results using a second marker that was either intragenic or immediately proximal to PKD1, but the closest known highly polymorphic marker was at least 70 kb away. In the course of performing mutation analyses, we discovered a polymorphic locus, EJ1, in exons 45–46 that is located approximately 1.6 kb proximal to KG8 (Figure 2). It has at least three allelic variants (A1–A3) that can be detected using heteroduplex analysis. We used this marker to test for

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**Table 1. Summary of Clonality Assay and LOH for Single Renal Cysts from ADPKD Patients**

<table>
<thead>
<tr>
<th>Patients</th>
<th>Number of Cysts Evaluated</th>
<th>Monoclonal Cysts Number</th>
<th>%</th>
<th>Cysts with LOH</th>
<th>KG8 Allele Lost</th>
<th>EJ1 Allele Lost</th>
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</thead>
<tbody>
<tr>
<td>JHU93</td>
<td>6</td>
<td>6</td>
<td>100</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>JHU188</td>
<td>9</td>
<td>7</td>
<td>78</td>
<td>ND</td>
<td>Ni</td>
<td>Ni</td>
</tr>
<tr>
<td>JHU244</td>
<td>12</td>
<td>10</td>
<td>83</td>
<td>ND</td>
<td>Ni</td>
<td>Ni</td>
</tr>
<tr>
<td>JHU245</td>
<td>10</td>
<td>5</td>
<td>50</td>
<td>C3</td>
<td>110 nt</td>
<td>A1</td>
</tr>
<tr>
<td>JHU246</td>
<td>13</td>
<td>12</td>
<td>92</td>
<td>C4</td>
<td>110 nt</td>
<td>A1</td>
</tr>
<tr>
<td>JHU288b</td>
<td>10</td>
<td>8</td>
<td>80</td>
<td>C8</td>
<td>104 nt</td>
<td>A2</td>
</tr>
<tr>
<td>JHU273b</td>
<td>13</td>
<td>11</td>
<td>85</td>
<td>C8</td>
<td>106 nt</td>
<td>A2</td>
</tr>
<tr>
<td>JHU304</td>
<td>3</td>
<td>3</td>
<td>100</td>
<td>ND</td>
<td>ND</td>
<td>Ni</td>
</tr>
<tr>
<td>Total</td>
<td>76</td>
<td>62</td>
<td>82</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*Number of cysts from which androgen receptor-specific PCR products could be amplified after HhaI or HpaII digestion.

* Samples for which the final rinses were tested for contamination.

ND, not determined; Ni, not informative; nt, length in nucleotides.
that cyst samples isolated from the same donor had loss of an identical allele (Figure 3C). A total of 30 cysts from three donors were evaluated with this marker, and 7 were found to have either a deletion or novel somatic mutation involving this locus (Table 1).

Loss of the Unaffected Haplotype
The concordance between the KG8 and EJ1 data strongly suggested that the lost alleles belong to the same haplotype. We confirmed this hypothesis using a method that exploited the physical proximity of the loci. A genomic fragment containing EJ1 and KG8 was amplified from the peripheral blood of each donor showing LOH for both markers using unlabeled A1 (B) and A2 (data not shown). All clones contained either KG8 allele 110 bp and EJ1 allele A1 (clones 1 and 3) or KG8 allele 106 bp and EJ1 A2 allele (clones 2, 4, and 5). nt, length in nucleotides.

A Novel Somatic Mutation
One cyst sample was discovered to have discordant results with KG8 and EJ1. This sample (C12) did not have loss of a KG8 allele but did have a unique heteroduplex pattern with EJ1 (Figure 3). Mixing studies determined that the A2 allele of EJ1, which was lost in the other two...
Molecular Basis of Focal Cyst Formation in PKD1

Figure 5. Linkage Analysis of Two Polymorphic Markers Linked to PKD1 in the Families of Donors JHU273 (A) and JHU246 (B)

PKD1 is linked to 3'HVR and KG8 with maximum lod scores of 1.34 and 0.32, respectively, at a recombination fraction of 0.0 for both markers in pedigree 1 (A). The posterior probability of linkage of the disease in this family to PKD1 is >95%. Two-point analysis of 3'HVR Figure 6. A 2 bp Deletion in C12 (D) creates a novel MwoI site and KG8 favors the haplotype shown in (A). In pedigree 2 (B), the maximum lod scores for 3'HVR and KG8 are 2.18 and 1.93, respectively, at a recombination fraction of 0.0. The genotypes D, A1 and A4 segregate with ADPKD in pedigree 1 (A) and pedigree 2 (B), respectively. In each kidney donor (arrows), it is the KG8 allele that does not segregate with disease which is lost in cysts demonstrating LOH. Affected genotypes are indicated by closed bars; deduced genotypes are shown in brackets. The pedigrees as shown do not include all family members. Triangles are used to protect the identity of participants.

Cysts (C8 and C14) from this donor, had been replaced by a novel allele (A4) in C12. We cloned both the A1 and A4 alleles of the EJ1 locus from this sample and determined the sequence of the novel allele (Figure 6A). Comparison of the sequence of A4 to that of A1 revealed two differences. The first is a 2 bp deletion (ΔC12) at positions 12,694–12,695 of the cDNA sequence (HUMP-KD1A, GenBank accession number L33243). The ΔC12 deletion is predicted to cause a reading frameshift resulting in a truncated protein. This mutation also creates a new MwoI endonuclease restriction site not present in the sequence of either the A1 or A2 alleles of EJ1 (Figure 6B). This site was used to confirm the presence of the deletion in the original C12 DNA sample (Figure 6C).
ADPKD is the phenotypic variability exhibited by family members who share a common mutation. The focal nature of renal cysts is an extreme example of this variability since all renal tubular epithelial cells within a kidney have an identical mutation. These features suggest that other factors are required for cyst formation. Identification of these other components may explain the pathogenesis of the disease and possibly provide new avenues for developing therapies.

To evaluate the molecular mechanisms responsible for cystogenesis, we developed a novel method for isolating epithelial cells from individual cysts. This technique minimizes contamination from other cell types and avoids biases resulting from the use of cultured cells. We have used this approach to analyze multiple kidneys from females and have shown, using two independent methods, that renal cysts in ADPKD are clonal in origin. Our data suggest that somatic mutation of the previously normal PKD1 allele is the second, rate-limiting step in cystogenesis and is likely to account for the focal formation of renal cysts. The rate at which one acquires these "second hits" probably plays a major role in determining the course of disease and probably explains much of its phenotypic variability. It may be speculated that a similar two-hit mechanism may also be responsible for causing the extrarenal manifestations of PKD1.

Loss of the normal allele of PKD1 in cystic tissue suggests a molecular recessive mechanism of disease similar to that seen with numerous tumor suppressor genes. If our model is correct, however, the frequency of "second hits" in cystic epithelia must be extremely high to account for the thousands of cysts that are observed. This is in contrast to the small number of tumors found in other inherited disorders of the kidney in which somatic mutation of the normal allele is required for disease. Wilms' tumor (Huff et al., 1991) and von Hippel Lindau's disease (Foster et al., 1994) are representative examples.

Our data suggest that there is indeed a high rate of somatic mutation in PKD1. This is best illustrated by sample JHU273 (Table 1). Of the 13 cysts that were shown to be monoclonal, 2 had loss of alleles for KG8 and EJ1; 1 had a unique 2 bp deletion; and 8 had no detectable mutation of the normal allele. The last group did not have a common X-chromosome inactivation pattern, suggesting that they had arisen from at least two independent progenitor cells (Figure 1). Assuming that the somatic mutations arose after X-chromosome inactivation was completed, a minimum of four independent mutations must have occurred in the 13 cysts that were examined. We cannot determine whether the two cysts with LOH for KG8 had identical mutations or were independent events since they had identical X-chromosome inactivation patterns. Cysts from other kidneys, however, that had a similar pattern of LOH for PKD1 markers had different X chromosomes inactivated, suggesting that independent events had resulted in LOH (data not shown). Formal proof of our hypothesis must await the development of methods that can be used for mutation analysis of the full length of the PKD1 gene.

The very high rate of somatic mutation predicted by our model is surprising since no known dynamic elements have been identified within the genomic sequence of PKD1 and since the adult kidney is thought to have a relatively low mitotic index. However, we have previously reported an extremely unusual 2.5 kb polypyrinidime tract within intron 21 that may be responsible for the gene's increased rate of mutation (The American PKD1 Consortium, 1995). Similar but much shorter elements present within other genes have been shown to undergo triple-helix formation both in vitro (Young et al., 1991) and in vivo (Rao et al., 1988). Wang et al. (1996) recently have shown that triplex formation induces mutagenesis in a mammalian cell culture system and demonstrated a requirement for excision and transcription-coupled repair in this process. The authors hypothesized that formation of the triple helix causes a stall in transcription that triggers gratuitous and potentially error-prone repair. They proposed that naturally occurring triple helices may similarly trigger repair and mutagenesis and thus constitute endogenous sources of genetic instability.

We postulate that the polypyrinidime tract within PKD1 may cause ongoing errors in its transcription-coupled repair that result in a high frequency of somatic mutation. This model can explain the multiplicity of second hits as well as the apparent development of new cysts throughout the lifetime of an individual. Likewise, the genetic instability possibly associated with this unusual genomic structure may be responsible for the high incidence of PKD1 within the population. If this element is proven to be responsible for the gene's mutability, PKD1 will be the first example of a disease that results from this novel mechanism of somatic mutation.

The indistinguishable clinical presentation of patients with PKD2 suggests a two-step process in this disease as well. Whereas the initial step is certainly a germline mutation of PKD2, the nature of the second event is not yet known. Presently it cannot be excluded that there may be an uncharacterized unstable element hiding within the genomic structure of PKD2 that results in frequent somatic inactivation in a manner analogous to that of PKD1. The relative infrequency of PKD2 argues against this hypothesis, however. The phenotypic similarity of the disorders may offer an alternative hypothesis. Investigators have postulated that the gene products of PKD1 and PKD2 may be interacting partners of a common pathway. It has been suggested that the function of PKD1 may be to regulate the activity of PKD2 (Mochizuki et al., 1996). If this is correct, somatic inactivation of PKD1 may be the second step that leads to clonal expansion in PKD2 and possibly other forms of ADPKD. This model predicts that the frequency with
which cysts form in PKD1, PKD2, and PKD3 will be determined by the rate of somatic mutation of PKD1. The identical number of renal cysts observed in the three disorders is consistent with this hypothesis.

In this study, we have determined that random inactivation of the normal PKD1 allele in somatic tissue is the likely molecular explanation for the observed clinical variability and the focal formation of renal cysts in the most common form of ADPKD. Both the high incidence of the disease in the population and the large number of second hits that must occur to account for the number of cysts observed suggest that unique features of the PKD1 gene structure may be responsible for its mutability. Our data suggest a molecular recessive mechanism of disease since both alleles are mutated in renal cysts. These findings have important implications for investigators interested in developing models systems and suggest that treatment strategies directed at replacement of polycystin may prevent cyst formation and end-stage renal disease.

**Experimental Procedures**

**Preparation of Cystic Epithelial Cells from a Single Cyst**

Cystic kidneys were processed within 24 hours of removal from the patient and were maintained at 4°C. The surface of the cyst was first rinsed with PBS and then its contents were drained by needle and syringe. The needle was left inserted in the cyst for the duration of the washing and incubation steps. The cavity of intact cysts was rinsed a minimum of three times with Ca²⁺- and Mg²⁺-free PBS. The last rinse from some cysts was collected and stored on ice. PBS containing 2 mM EDTA was then injected into the lumen of the intact cyst. After a 20 min incubation, the cyst was massaged several times to assist in detachment of the epithelial cells from the basement membrane. Cysts that maintained the extraction solution (PBS/EDTA) in their lumina for the duration of the incubation period were considered intact. Only the epithelial cells of intact cysts were harvested by drainage. The cystic epithelial cells in the extraction solution (PBS/EDTA) and the last wash solutions were centrifuged at 1500 rpm (Beckman) for 15 min. The pellet was used for DNA preparation using the Puregene DNA extraction kit (Gentra) according to the manufacturer’s protocol. To assist in DNA precipitation, 10 μg of glycogen was added to each sample.

**Clonality Assay**

The DNA samples were digested with 10 units of HpaII or HhaI in and 45 affected individuals. The PCR products that gave each pattern were cloned into pCRII (Invitrogen) and then sequenced to determine the number of second hits that must occur to account for the number of cysts observed. Heteroduplex analysis was performed as described above. GenBank accession number M21743, and KG8R8, 5'-GCAAGGACCCAGCAGTCCGAG-3' (bp 13,014-14,034), 5 nM 32P-end-labeled KG8F8, and 1.5 mM MgCl₂. The PCR products were diluted with 5 μl of volume of sequencing loading buffer and separated in a 6% denaturing polyacrylamide gel. The dried gel was examined by autoradiography using X-Omat XAR film (Kodak) at −80°C overnight with an intensified screen.

**Loss of Heterozogosity Assay Using KG8**

The microsatellite KG8 that is present in the 3' UTR of PKD1 mRNA was used to distinguish the mutant and normal alleles (Snary et al., 1994). Cystic DNA samples served as template for PCR amplification. PCR amplification was performed for 28 cycles using an identical protocol as that used for the androgen receptor except that the reaction included a final concentration of 1 μM of each KG8 primer (KG8F8, 5'-CTCCAGGGTTGAGGAAAGTTG-3' [bp 13,925-13,945], HUMPKD1A, GenBank accession number L33243) and KG8R8, 5'-GCAAGGACCCAGCAGTCCGAG-3' (bp 13,014-14,034), 5 nM 32P-end-labeled KG8F8, and 1.5 mM MgCl₂. The PCR products were diluted with 5 μl of volume of sequencing loading buffer and separated in a 6% denaturing polyacrylamide gel. The dried gel was examined by autoradiography using X-Omat XAR film (Kodak) at −80°C overnight with an intensified screen.

**Heteroduplex Analysis**

Either 200 ng of genomic DNA (isolated from whole blood using the Puregene kit) or 5 μl of cyst DNA was used as template for amplification of a 540 bp product (EJ1) using primers FQF28 (5'-ACGCCTTGCCGTGAGG-3', 12,539-12,556; HUMPKD1A) and FQR35 (5'-ATGGGACCGGAGAGATCC-3', 12,977-12,995). PCR was performed as follows: denaturation at 94°C for 5 min; 35 cycles of 94°C for 30 sec, 62°C for 30 sec, and 72°C for 30 sec; and a final extension of 72°C for 10 min. The total PCR volume was 30 μl using 2 μl of Taq DNA polymerase (Boehringer Mannheim), 0.2 μl dCTP, and a final MgCl₂ concentration of 1.5 mM. Heteroduplex analysis was performed using Hydrolink Mutation Detection Enhancement gels (MDE, AT Biochem) following the manufacturer’s protocol. Urea was added to the gel to a final concentration of 15% to minimize band broadening. The radiolabeled PCR products were initially denatured by heating at 95°C for 5 minutes and then allowed to cool to room temperature gradually over 1-2 hr before loading. Gels were run at 700 V for 14-16 hr, dried, and placed on X-Omat XAR film (Kodak) at room temperature or on a Phosphomager cassette (Molecular Dynamics).

**Mixing Studies**

The polymorphic locus EJ1 was identified by heteroduplex analysis using the primers FQF28-FQR35 as described above. This PCR product spans part of exons 45 and 46 as well as the 90 bp intron between them. PCR was performed for 28 cycles of 94°C for 20 sec, 65°C for 20 sec, and 72°C for 30 sec; and a final extension of 72°C for 10 min. The total PCR volume was 30 μl using 2 μl of Taq DNA polymerase (Bio-Rad Laboratories), and 2 mM MgCl₂. The sequences of the primers were PCR conditions described above. GenBank accession number L33243), whereas A2 had a conservative base pair change (C-to-T) at position 12,617, which did not alter the amino acid sequence (leucine, amino acid 4136). A3 had eight consecutive guanines in intron 45 instead of the seven contained in the published sequence (HUMPKD1A, GenBank accession number L33243), and KG8R8, 5'-GCAAGGACCCAGCAGTCCGAG-3' (bp 13,014-14,034), 5 nM 32P-end-labeled KG8F8, and 1.5 mM MgCl₂. The PCR products were diluted with 5 μl of volume of sequencing loading buffer and separated in a 6% denaturing polyacrylamide gel. The dried gel was examined by autoradiography using X-Omat XAR film (Kodak) at −80°C overnight with an intensified screen.

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The 2 bp deletion identified in the C12 mutant clones created a new MwoI site. To test for the presence of this site, a nested 172 bp PCR product was amplified using primers C12F1 (5'-TGCCGTTGATCCAC-3' and the androgen receptor locus (so that the alleles could be distinguished) was added to each cyst DNA sample prior to PCR to control for the completeness of HpaII or HhaI digestion. In most cyst samples, the male-specific product was not amplified after HpaII or HhaI digestion, as expected.

**Molecular Basis of Focal Cyst Formation in PKD1**

985
References


