Loss of heterozygosity in polycystic kidney disease with a missense mutation in the repeated region of \textit{PKD1}

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**Abstract** Loss of heterozygosity (LOH) is a molecular phenomenon that denotes the loss of one of the two alleles at a specific locus. It is frequently associated with tumour suppressor genes in various cancers and also with hyperproliferative disorders, although not exclusively. Interestingly, in conditions where there is an inherited germline mutation, the lost allele is always the functional one, thereby rendering a phenotypically dominant disease of recessive character at the cellular level. A disease more recently shown to be associated with LOH is polycystic kidney disease type 1, a systemic disorder characterized by significant pleiotropy. The main pathology is from renal cyst formation that eventually leads to end-stage renal failure during adult life. We describe the identification of a missense mutation in the repeated part of the \textit{PKD1} gene, exon 31, that substitutes valine for methionine. The mutation, M3375V, cosegregates with the disease phenotype in a large Cypriot family. During transplantation of one patient, one of the polycystic kidneys was removed and DNA was isolated from cystic epithelial cells. In 3 of 17 cysts examined with intragenic and flanking polymorphic markers on chromosome 16 we detected LOH, since the wild-type allele was lost, thereby rendering the affected kidneys of mosaic character. The degree of LOH was extensive and varied among the three cysts, supporting the multiplicity of expression of the phenomenon on different occasions. No LOH was detected for other selected loci examined. Our work further supports the hypothesis that the rate-limiting step in cyst formation may be the occurrence of a second somatic hit, although other factors may be also involved. The high frequency of mutations at this locus may, to a great extent, explain the variability in phenotype observed among patients in the same families, and the relatively high frequency of the disease worldwide.

**Introduction**

The autosomal dominant form of polycystic kidney disease (ADPKD) is one of the most frequent inherited disorders, affecting approximately 1/1000 individuals. It is the most frequent inherited renal cystic disease, mainly characterized by the formation of fluid-filled cysts in about 5% of nephrons. The cysts grow in size and number and with time they destroy the renal parenchyma, leading to end-stage renal disease (ESRD). It is a systemic pleiotropic condition, also affecting other organs such as liver, pancreas and the vascular system. An early common feature is hypertension, which in turn complicates the patient’s condition (Dalgaard 1957; Gabow 1993).

One interesting characteristic is the interfamilial and intrafamilial phenotypic variability (Parfrey et al. 1990; Bear et al. 1992; Gabow et al. 1992; Ravine et al. 1992; Coto et al. 1995), which is partly explained by genetic heterogeneity, since a mutation in any one of three different genes has been shown to cause essentially indistinguishable symptomatology. Most frequent, accounting for 85–90% of cases (Peters and Sandkuijl 1992), is \textit{PKD1}, which maps on chromosome 16p13.3; its complete sequence has been obtained (European PKD Consortium 1994; Burn et al. 1995; Hughes et al. 1995; International PKD Consortium 1995). \textit{PKD2} on chromosome 4 has also been cloned and sequenced and accounts for 10–15% of cases (Mochizuki et al. 1996), whereas \textit{PKD3} is still unmapped and accounts for few families so far (Daoust et al. 1995). The product of the \textit{PKD1} gene, polycystin 1, is one of the largest proteins characterized, with 4302 amino acids encoded in 46 exons. It consists of many diverse domains, which include leucine-
rich repeats, a C-type lectin domain, immunoglobulin (Ig-like) repeats, and transmembrane regions. According to a recent model of the protein, 3109 amino acids are in the extracellular N-terminal domain, whereas a rather short C-terminal domain of 199 residues is cytoplasmic (Sandford et al. 1997).

Seventy per cent of the 5′ end of the gene is repeated elsewhere on chromosome 16 with homology that approaches 95% (European PKD Consortium 1994), a fact that impedes mutational analysis of the gene. Its sequence is GC-rich and it contains the largest known polypyrimidine tract, of about 2.5 kb, which is considered partly responsible for the high mutability of the gene (Qian et al. 1996; Watnick et al. 1997). Recent experiments have shown that polycystin 1 contains a coiled-coil C-terminal cytoplasmic domain, which can interact in vitro with the corresponding cytoplasmic domain of the PKD2 gene product (Qian et al. 1997; Tsiokas et al. 1997). These findings suggest that the two proteins play a role in a common metabolic pathway that is destined to transfer signals from the extracellular matrix into the cell.

Part of the clinical variability in this disease, and in each individual kidney in particular, has been recently attributed to the phenomenon of loss of heterozygosity (LOH; Qian et al. 1996; Brasier and Henske 1997). The findings suggested that only a small number of nephrons become cystic, simply because only these undergo a second somatic hit eliminating the normal PKD1 allele during life. Apparently this second hit triggers cyst formation, since the affected cells remain with a single allele, which is the inherited mutated copy. This phenomenon, at the same time, establishes mosaicism in the cystic kidneys, which contain two different types of cells: those that are heterozygous and those that are hemizygous for the inherited germinal mutation.

In this report, we provide evidence supporting the above hypothesis, by identifying a germline mutation in the duplicated part of the gene that cosegregates with the disease in a large family and is associated with LOH. Out of 17 cysts investigated from a single kidney surgically resected from a transplanted patient, three showed loss of markers intragenic to, or flanking the PKD1 gene. This is only the third report documenting LOH in ADPKD1 and also raises the question what is the event triggering formation of the cysts not showing LOH.

**Materials and methods**

**Patient**

The patient belonged to a family, CY1616, that was previously investigated by DNA linkage analysis (Constantinou Deltas et al. 1996). The family showed evidence for linkage to the PKD1 locus with markers KG8 and SM6. The two-point lod scores were 1.25 with both markers. The index patient reached ESRD and, without commencing haemodialysis, he was transplanted at the age of 41 years with a kidney donated by his healthy brother. At the time of transplantation one polycystic kidney was resected and placed on ice. Cyst epithelial cells were isolated within the next 2 h.

Isolation of cyst epithelial cells and genomic DNA extraction

Essentially the procedure described by Brasier and Henske (1997) was followed. Well-separated cysts were chosen for analysis. Single cysts were dissected, removed and rinsed in excess of PBS prior to the isolation of epithelial cells. Cells from 17 individual cysts were directly isolated by scraping the inner cystic surface with a razor blade, leaving the cyst wall intact. Prior to DNA isolation, the epithelial cells were sedimented and washed twice with PBS. Finally, they were resuspended in 100 µl of a solution containing 50 mM KCl, 10 mM Tris-HCl, pH 8.5, 1.5 mM MgCl2, 100 µg/ml bovine serum albumin and 100 µg/ml protease K. After overnight incubation at 37°C the mixture was sequentially extracted with 1:1 phenol:chloroform and 24:1 chloroform:isoamyl alcohol and the DNA was ethanol/salt precipitated. The genomic DNA was resuspended in 50 µl of H2O and 2 µl was used for the polymerase chain reaction (PCR).

Long-range PCR amplification (LR-PCR), single-strand conformation polymorphism analysis (SSCP) and DNA sequencing

Genomic DNA isolated from whole blood or from cysts was used as template for amplification of a 6.5-kb fragment. The primers were located in exons 23 and 34. Three multicolline was used for a second-round booster PCR with the reverse primer remaining the same, whereas a nested forward primer also located in exon 23 was used (Fig. 1, Table 1). LR-PCR was performed according to a modification of the method of Barnes (1994). Briefly, PCR buffer consisted of 20 mM TRIS-HCl, pH 8.75, 16 mM (NH4 )2 SO4, 150 µg/ml bovine serum albumin, 8% glycerol, 2.5 mM MgCl2 and 100 µM each dNTP. DMX-SO, to a final concentration of 7%, and 1.5 µl 2 M Trizma-base were also included in the 100-µl PCR mix. A hot-start protocol was used with the enzyme mix added at 80°C. The enzyme mix consisted of 2.4 U of TaqExpress (GenPak, Brighton, UK) and 0.1 U of pfu polymerase (Stratagene, La Jolla, Calif., USA). PCR cycling was as follows: denaturation at 94°C for 1 min, 35 cycles of 94°C for 8 s, 58°C for 30 s and 68°C for 16 min.

Exon-by-exon screening for mutations was performed on the LR-PCR product by SSCP analysis. The LR-PCR product was first diluted 100-fold. Sequences of primers and conditions for PCR are summarized in Table 1. One microtitre of the diluted LR-PCR product was used as template in a total volume of 10 µl. SSCP analysis was performed as previously described (Neophytou et al. 1996). Exons showing novel SSCP patterns were directly sequenced using the Sequenase sequencing kit from USB (Amersham, Cleveland, Ohio, USA) according to the manufacturer’s instructions.

Demonstration of LOH

For the demonstration of LOH, several informative polymorphic markers were examined that represented short tandem repeats at various loci. Markers on chromosome 16 were IV342 (intragenic) (Peral et al. 1996a, b; Constantinides et al. 1997), HBAP1, SM6, D16S403, D16S753, D16S771 and D16S539. Other markers were D4S234, linked to PKD2 on chromosome 4, D17S1298 and D17S849, linked to tumour suppressor gene p53 on chromosome 17, D8S179, linked to c-myc on chromosome 8, and D3S2409 on chromosome 3 (3p21.2–21.3). This marker was tested because of its previously reported association with LOH (Brasier and Henske 1997). Except for polymorphism IV342, which is easily analysed by simple agarose gel electrophoresis, all other genotypes were analysed using a forward oligonucleotide primer that was end-labelled with [γ-33P]ATP and T4 polynucleotide kinase, and the resulting PCR products were electrophoresed on a urea denaturing polyacrylamide sequencing gel. LOH was determined by visual comparison of autoradiographic signals obtained from amplification of cyst and control DNA from the patient’s blood.