MUTATION IN BRIEF

Novel Splicing and Missense Mutations in Autosomal Dominant Polycystic Kidney Disease 1 (PKD1) Gene: Expression of Mutated Genes

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Autosomal dominant polycystic kidney disease (ADPKD) is a common disorder mostly characterized by cyst formation in kidney tubules. The majority of ADPKD cases is caused by mutations in the PKD1 gene, but no prevalent mutation has been reported. By heteroduplex analysis of the 3' single-copy region of the gene, we have searched for mutations in subjects from 40 ADPKD families of Northern Italy. Seven novel polymorphisms and three novel disease-associated mutations (R3718Q, L3851P and IVS45+56del25) were identified. Both missense mutations are located in the major extracellular loop of polycystin-1. The 25 bp deletion inside intron 45 did not affect 5' and 3' consensus splicing sites, but caused a 56 nucleotide out of frame-deletion due to activation of a cryptic 3' splice site in exon 46. The mutated RNA should produce a truncated polycystin 1 at the G binding peptide in the intracellular C-terminal end of the protein. RT-PCR analysis showed that the disease-associated mutations were present in transcribed sequences. In particular, RNA analysis of BHK cells transfected with PKD1 genomic DNA, including the deleted intron, showed that no normal transcript is produced by the deleted gene. This intronic mutation, found in a large pedegree, seems to be associated with a prevalence of cerebrovascular disease. © 2000 Wiley-Liss, Inc.

KEY WORDS: polycystic kidney disease, autosomal dominant; PKD1; ADPKD; splicing and missense mutations

INTRODUCTION

Autosomal dominant polycystic kidney disease (ADPKD; see MIM# 173900) is the most common single gene disorder (1/1000) leading to kidney failure; more than half of affected individuals require renal replacement therapy by their sixth decade, accounting for 10% of the renal dialysis population (Gabow, 1993). Approximately

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85 % of ADPKD is linked to the PKD1 gene (MIM# 601313) on chromosome 16p13.3. The gene encodes a 4302 amino acid membrane glycoprotein, polycystin-1, with a large N-terminal extracellular region, 11 putative transmembrane helixes, and a C-terminal cytoplasmic domain of 226 amino acids, recently implicated in signal transduction (Harris, 1999). In the extracellular and membrane associated region there is a sequence similarity over ~2000 amino acids with the human receptor for egg jelly related (PKDREJ; MIM# 604670) protein (Hughes et al, 1999).

Most of the remaining ADPKD cases are linked to the PKD2 gene (MIM# 173910), on chromosome 4q encoding a 968-aminoacid integral membrane protein, polycystin-2, with six predicted membrane spanning domains and intracellular N- and C-termini (Mochizuki et al, 1996); polycystin-2 is similar to part of the membrane associated region of polycystin-1 and also shows homology to alpha subunits of voltage-gated calcium and sodium channels. Recently, PKD2L and PKD2L2 genes have been found, structurally closely related to polycystin-2 and with comparable degree of similarity with polycystin-1 (Wu et al, 1998; Veldhuisen et al, 1999). PKD2L acts as a selective cation channel (Chen et al, 1999), but is not associated with renal cystic disease. This information and the fact that polycystin-1 and -2 associate physically in vitro with their predicted cytoplasmic COOH domains (Qian et al, 1997), indicate that they may be partners in a common signaling cascade involved in tubular morphogenesis in which the translocation of Ca ions acts as a second messenger. Therefore, abnormal ion regulation caused by specific PKD mutations may trigger the cellular changes associated with cystogenesis.

The mutation rate in the PKD 1 gene is significant (Peral et al, 1997; Roelfsema et al, 1997). However, the number of mutations so far reported is limited, because the mutation detection is hampered by the genomic structure of the 5' region of the gene (Harris, 1999) which is repeated several times elsewhere on chromosome 16. In addition, most mutations are unique to a single family, each population containing many different mutations (Harris, 1999). Therefore, detection of PKD1 gene mutations is important for diagnostic reasons, but also because they can provide insight into the disease mechanism and identity of functionally critical areas of the polycystin-1.

The incidence of the subjects requiring dialysis with ADPKD in the region of the Po river delta, Northern Italy, appeared to be more than twice the average reported for the European population (del Senno et al, 1988). We screened 40 unrelated ADPKD subjects from this region, for PKD1 mutations within the 3' region of the gene. We have identified possible disease causing mutations in three families and seven novel polymorphisms. Two are missense mutations and the other is a splicing aberration caused by a deletion in the intron 45. To our knowledge, these are the first missense mutations in the large extracellular loop of PKD1 protein, and the first intronic deletion involving the polypyrimidine region and leading to aberrant splicing. The possible relation between aberrant splicing, small intron size and polypyrimidine stretch is discussed.

MATERIALS AND METHODS

Family selection, and clinical information

Forty unrelated ADPKD individuals of Italian origin were included in this study. When family studies were available, linkage analysis with microsatellite markers close to PKD1 gene was used to confirm that the ADPKD families studied had PKD1 disease. We cannot exclude that a small number of patients have PKD2 disease. All the patients investigated were informed of the study.

PKDFe3. ADPKD could be traced through six generations with end stage renal disease (ESRD) in the proband (female) at age 47 years and in other 4 subjects (ages 47, 48, 49 years - males - and 56 years - female -). In the third generation, three female subjects out of 8 affected subjects, died of vascular cerebral events; in the fourth generation, in two out of four affected subjects, hemorrhagic ictus and subaracnoidal hemorrhage (the proband) were reported. Her affected daughter, 36 years old, was hypertensive with renal function within the normal range.

PKDFe18. ADPKD could be traced through two generations with ESRD onset at age 61 years in the proband. Her affected 60 year old sister was hypertensive and with mild renal insufficiency. The two affected daughters, age 35 and 33 years, had normal renal function without hypertension.

PKDFe33. ADPKD could be traced through three generations. The proband, 67 years old, was hypertensive and with mild renal insufficiency. Her affected daughter (41 years) and son (36 years) had renal function within the normal range with hypertension. Her affected niece (26 years) had renal function within the normal range, without hypertension.

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Isolation and analysis of DNA and RNA

DNA was extracted from peripheral blood (PB) cells by standard procedure or from Epstein-Barr virus-transformed (EBV-transformed) lymphoblastoid cells (Aguiari et al, 1999). The search for DNA variations was performed by heteroduplex analysis (Aguiari et al, 1999). Briefly, PCR primers in the 3' unique region of the gene, reported in table 1 and originally produced for cDNA analysis, were used for DNA amplification as previously described (Peral et al, 1996).

TABLE 1 : Oligonucleotide Primers for Amplification of the Human PKD1 Gene

Exon	cDNA (nt)	5' primer	cDNA (nt)	3' primer
36	10609	cagGACTGGTGGAGGGTCT	10815	cctcacCTTCAGTGGCTCC
36-37	10775	GCGCCAGCTTCCTGGCCTCATTC	10956	TGCAAAGCCGTGGGGTGGCCGTA
37	10818	gGTCTTGCTGGAAGCCCTGTAC	11007	CATGCCATGTAGCCTCTTGACC
37-37I	10940	CACCCCACGGCTTTGCAC		actgcagtggtgcttaggg
37I-38		actcctgcctggccctgaa	11105	CTTTGCAGACGGTAGGCGTG
38-39	11028	CATGCTTTTTCTGCTGGTGACC	11227	GCTCTGGGCTGGACTGGTTC
40-41	11323	TTCAGCACCAGCGATTACGAC	11535	cCTGTTGTCCAGCCAGTTGTG
41-42	11510	AGCTGCACAACTGGCTGGAC	11709	cCGAGGTGAGCAGAGGCAG
43-45	11909	CGCCGCTTCACTAGCTTCGAC	12171	ACGCTCCAGAGGGAGTCCAC
45	12182	TGTTGGTGCTGTGCCCTGGGACT	12399	CAGGAACAACTCCACCATCTCGT
45-46	12303	GGCTGTTATTCTCCGCTGGCGCT	12480	CAGCGGCTCCATCCCTTCAAAGC
46'	12442	agTTCCGCCACAAAGTCCGCT	12654	GAACACGGCTTGGAGGCGGGAGG
46''	12608	TGGGGACAAGGTGTGAGCCTGAG	12786	GGGATGGGCCACGGGAAGATCCG
46'''	12730	AGCCTGCAAGGCCGCAGGAGCAG	12965	TAATACTGAGCGGTGTCCACTCC

PCR products were analyzed on nondenaturing Hydrolink Mutation Detection Enhancement gels (MDE, AT Biochem) following the manufacturer's protocol. Sequences from exons 36-46 were screened to 80 %, as shown in Fig. 1A. PCR products showing an abnormal pattern were directly sequenced.

Cytoplasmic RNA, obtained from either peripheral-blood lymphocytes or EBV-transformed lymphoblast cell lines, was reverse transcribed by using the SuperScript Preamplification System (Life Technologies) and the 5'CTAGAAACCGTCCAATACTGCTGTGTCCTTC3' reverse primer located in exon 46, 3' to the stop codon. cDNA amplification was performed with reverse and forward primers indicated in table 1 for exons 38-39 and 41-42. When specified, amplified DNA and/or cDNA were directly treated with restriction enzymes, and analyzed by agarose and/or polyacrylamide gel electrophoresis.

Recombinant plasmid preparation, cell culture and transfection

PKD1 DNA of a subject carrying the IVS45-25del mutation was amplified from Ex 43 to Ex 46 by using restriction site-tailed primers (BamHI, 5'CAGGATCCATGCGCCGCTTCACTAGCTTCG ACC3') and (EcoRI, 5'CAGAATTCTAATACTGAGCGGTGTCCA3') as previously reported (Aguiari et al, 1998). Restricted fragments were cloned in the mammalian expression vector pCDNA3, as described (Aguiari et al, 1998). Clones with normal IVS 45 and those with the 25 bp-deleted IVS 45 were characterized by PCR analysis, as described in the legend of Fig. 2.

BHK-21 cells, plated (20-40 % confluence) on 100 mm dishes 16-20 h before treatment, were transfected with 10 μg of plasmid DNA. At 24 h after transfection, cells were rinsed rapidly in phosphate-buffered saline (PBS) and scraped in RNAzol lysing solution (Rossi et al, 1996). Total RNA was extracted using the RNAzol kit and treated with DNAse as previously described (Rossi et al, 1996). PKD1 RNA sequences were analyzed by RT-PCR with primers reported in the legend of Fig. 2.

RESULTS

Mutations

In family PKDFe-18, the substitution 11552 T → C in exon 42 (producing the heteroduplex of case 5 in Fig. 1B) converted Leu 3851 to Pro (L3851P). The missense mutation, found in a 61-year-old female patient with ESRD, created a MspI restriction site that was used to confirm that the two affected daughters had inherited this change (Fig. 1C). No other example of this transition was observed in our series of 100 investigated chromosomes. RT-PCR of total RNA isolated from peripheral-blood lymphocytes with primers in exons 41 and 42 and MspI

restriction analysis (Fig. 1D) showed that the mutation was also present in transcribed sequences, and that the mutated allele was expressed.

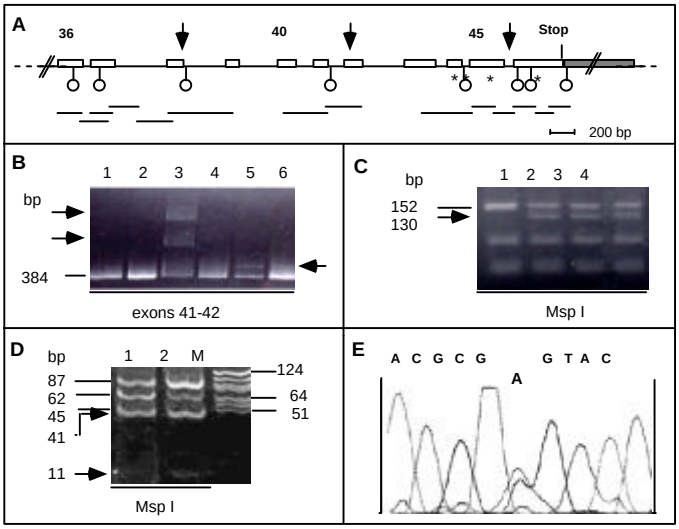

Figure 1: Characterization of mutations in the unique region of the PKD1 gene. A: PKD1-gene region that has been amplified by PCR, shown as fragments of different sizes. New and reported polymorphisms are indicated by circles, and asterisks, respectively. Disease-associated mutations are indicated by arrows. B: Heteroduplex analysis of 41-42 PCR fragments in 6 PKD subjects (1-6). Fragments were amplified and analyzed as described in the method section. Heteroduplexes are indicated by arrows. C: MspI restriction analysis of the T to C mutation at position 11552 in the PCR DNA from a non-affected brother (1) and in the two affected daughters (2, 3) of the PKD propositus (4). The mutation introduces a new Msp1 site producing the 130 bp fragment. D: MspI restriction analysis of the T to C mutation at position 11552 in the cDNA from a non-affected brother (1) and in the PKD propositus (2). In the mutated cDNA fragment, Msp1 cuts the 62 bp fragment in two fragments of 41 and 11 bp in size. E: Direct sequence analysis of the PKD subject with the heteroduplex in PCR fragment 38-39 exons. The G to A mutation is at position 11153.

Sequence alignments of human PKD1, PKDREJ, PKD2 (Hughes et al, 1999), PKD2L (Wu et al, 1998) and PKD2L2 (Veldhuisen et al, 1999) genes and murine PKD1 gene (Lohning et al, 1997) revealed that this leucine was conserved in the first two genes, and was substituted by valine in the murine PKD1 and by isoleucine in the human PKD2 genes.

In family PKDFe-33, the nucleotide at position 11153 at the end of exon 38 was changed from G to A (see Fig. 1 E), converting Arg 3718 in Gln (R3718Q). The missense mutation, found in a 67-year-old woman with a mild chronic renal disease, caused the loss of a BstUI restriction site, and this enzyme was used to show the same modification in the affected daughter, son and niece, as well as the RNA expression of the mutated sequence (data not shown). This mutation was not found in non affected members of the family and in our series of investigated chromosomes. Sequence alignments of human and murine PKD1, and the other members of the human PKD family genes revealed that this aminoacid is conserved in murine PKD1, but substituted by the basic lysine in the PKDREJ gene, and by serine in the PKD2, PKD2L and PKD2L2.

In family PKDFe-3, a 25-bp deletion (between pairs +53/+56 and +77/+80) was found within intron 45: PCR products with primers for exons 45-46 produced two distinct fragment sizes on agarose-gel electrophoresis in all affected members of the family (Fig 2A), showing that the mutation co-segregated with the disease.

RT-PCR on total RNA isolated from peripheral-blood lymphocytes of carrier subjects, with primers in exon 45 and 46, produced an additional fragment smaller than the expected normal one (Fig. 2B, left). DNA from exon 43 to 46 was cloned in pCDNA3 expression vector, and normal and mutated cloned sequences were transfected in BHK cells. RT-PCR analysis showed that in cells transfected with the mutated sequences only the small allelic fragment was found (Fig. 2B, right). Therefore, no normal PKD1 mRNA could be obtained from the mutated allele. Direct sequencing of the low-size fragment revealed that there was an abnormal splicing because a cryptic 3' splice site at position +56 of exon 46 had been activated. This resulted in a 56 bp deletion of coding sequences, and a premature stop at the new codon 4189 was created, as summarized in Fig. 2C).

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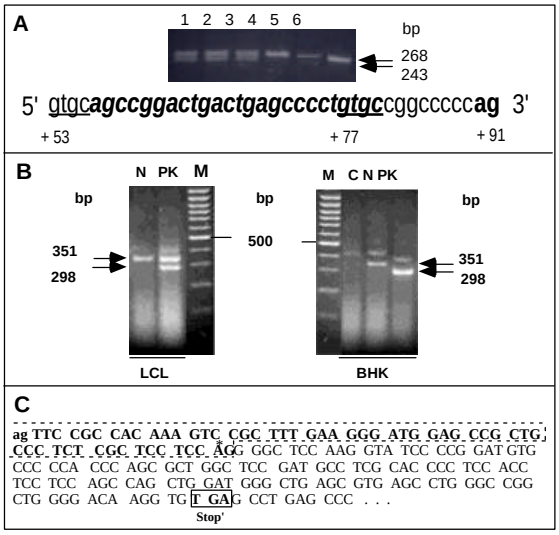

Figure 2: Characterization of the intronic deletion detected in the PKDFe-3 family. A: PCR fragments were obtained from PB cell DNA of three PKD affected and non-affected family members. The deleted seqence is indicated in bold italic characters. The repeat gtgc is underlined. The ag 3' splice sequence is included. B: RT-PCR analysis of PKD1 sequences encompassing intron 45. On the left, the amplification of cDNA obtained from lymphoblastoid cells (LCL) of a control (N) and the affected subject (PK). On the right, the amplification of cDNA obtained from BHK cells transfected with the plasmid containing the normal allele (N) and with the plasmid containing the mutated allele (PK). C indicates cells transformed with the empty plasmid vector. cDNA was produced as described in the Methods section, and amplified with exon 45-46 forward and 46' reverse primers, indicated in table 1. C: Cryptic 3' splicing site and frameshift mutation produced by the intronic deletion. The abnormally spliced exonic sequences are included in the dotted line, the new ag 3' splice sequence is indicated by the asterisk, and the premature stop codon is boxed.

Polymorphisms

A number of other substitutions were detected (Table 2), which proved to be polymorphisms either because they do not change the encoded aminoacid, or by family studies.

Neutral polymorphisms include the 10765C→T (L3589L), which was recently reported as a C→A substitution (Perrichot et al, 1999), 10869G→A (P3623P), 12444 C→T (F4148F), 12627T→C (P4209P). In addition, after the canonic stop codon, a 12922 G→A was found in PKD and normal subjects of one family. A C→A substitution in intron 44 (IVS44,+44) was observed only in one PKD individual, and no other member of the family was studied, but the nucleotide variation seemed to be too far from the splice site to be a mutation.

Other reported polymorphisms were found: the 12130A→G (I4044V) (Daniells et al, 1998); 12271 A→G (Peral et al, 1996), A→T and A→C (A4091A); 12762 C→T (P4254P); the deletion of a G in intron 44 (IVS44+19delG) (Daniells et al, 1998), and the G→A substitution in intron 38 (IVS38+13 G→A) recently reported (Perrichot et al, 1999) which was found only in one case.

The GAG insertion at +8 in intron 41 (IVS41+8insGAG) (producing the heteroduplex of case 3 in Fig. 1 B) was found in one woman who reached ESRD at 43 years old. The same insertion was found in her affected brother. No further family DNA sample was available.

DISCUSSION

This analysis of the 3' part of the PKD1 gene has shown two novel missense mutations, one novel splicing mutation in intron 45 and seven new and five reported polymorphisms.

Most of the mutations found in the PKD1 gene seem to be unique (Harris et al, 1999) and result in premature stop of translation. These mutations are clearly pathogenic. Missense mutations are less frequent (20 out of 80 mutations so far reported (Peral et al, 1997; Roelfsema et al, 1997, Watnick et al, 1997; Daniells et al, 1998; Afzal et al, 1999; Badenas et al, 1999; Perrichot et al, 1999; Thomas et al, 1999). Their definition as pathogenic mutations may remain uncertain until the rest of the gene can be completely screened and until definitive functional assays can be performed.

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TABLE 2.: Mutations and Polymorphisms of PKD1 Identified in This Study

Exon	Change	Effect	Restriction	frequency
		Polymorphisms		
36	C10765T	L3589L	- NlaIV	0.13
37	G10869A	P3623P	- MspI	0.04
44	A12130G	I4044V	- FokI	0.18
45	A12271T	A4091A	+ BanII	0.04
"	A " G	"	+ HhaI	0.22
"	A " C	"	+ NlaIV	0.04
46	C12444T	F4148F	- AciI	0.10
46	T12627C	P4209P	- DdeI	0.08
46	C12762T	P4254P	- AciI	0.08
46	G12924A		- AciI	
IVS				
38	G>A +13			
41	GAG-ins +8			
44	del G +19		+ BglI	0.12
44	C>A +44			
		Disease-associated mutations		
Ex 38	G11153A	R3718Q	- BstUI	
Ex 42	T11552C	L3851P	+ MspI	
IVS 45	25 bp-del +55			

The effect of the missense mutations described here could be deduced by the conservation of the aminoacids during evolution. The missense mutations result in proteins with the loss of a positive charge in the case of R3718Q and, in the case of L3851P, a predictable alteration in the secondary structure of the molecule disrupting α helixes or β strands. To our knowledge, these are the first missense mutations found in the major extracellular loop of polycystin-1, between TM6 and TM7, where one stop and in frame deletions have been reported (Peral et al, 1996; Afzal et al, 1999). This region, corresponding to the first extracellular loop of the PKD2 proteins, has a high similarity between members of the PKD protein family (Harris, 1999). In particular, L 3851 is located inside a 23 aa sequence, which is highly conserved in PKD1 (aa 3840-3862) and PKDREJ protein (Hughes et al, 1999; Wu et al, 1998). Furthermore, the conservation of the aliphatic residue (L, V or I) among all proteins suggests that two methyl groups are potentially important for protein structure or function. The change R3718Q is in a less evolutionary conserved sequence of this extracellular loop in PKD1 (aa 3706-3727) which is not conserved in PKD2 and PKDREJ. The positive charge is conserved in PKDREJ protein, but not in PKD2 and PKD2L proteins. The fact that transcripts are observed with these mutations excludes the presence of mutations affecting RNA expression in the non investigated regions of the gene.

The 25 bp deletion inside the 91 bp intron 45 results in an abnormal splicing with excision of a region including 53 bp of exon 46, due to the activation of a cryptic splice site at codon 4166 (AGG). This is the fourth AG after the canonic AG splicing sequence, but this is the first with a polypyrimidine stretch in front (21 nucleotides with 13 C, 5 T and 3 G; see Fig 2 C).

Three different splicing mutations induced by intronic deletion have previously been reported in the PKD1 gene. Two deletions of 18 and 20 bp, respectively, were found in the intron 43 (Peral et al, 1995). Three different transcripts were produced in each case: one larger and two shorter than the normal, due to intron inclusion, activation of an upstream intron splice site or exon skipping. The most likely explanation of why these deletions cause aberrant splicing was related to the size of the intron itself which was reduced to 55 or 57 bp. It was proposed that, in such a small intron, the 5' and 3' splice sites were too close to allow the spliceosome complex to form. The third reported intronic deletion, was a 19 bp deletion in intron 31 (Peral et al, 1997), causing an aberrant splicing with inclusion of the mutated intron. The aberrant splicing seems more related to the loss of specific sequences than to a short exon size. Interestingly, the sequences deleted in both intron 31 and 43 contain four G triplets, and this repeat is a common component of human 5' splice site, characteristic of small introns and important for the correct 5' splicing (Mc Culloug and Berget, 1997).

The 25 bp deletion reduces intron 45 from 90 to 65 bp. This size, which corresponds to the size of the normal intron 19, could not be sufficient to justify the abnormal splicing. On the other hand, the deletion, removing a sequence including 5 pyrimidines from the 16 pyrimidine-rich stretch, and 3 adenines which might be branch

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point sites (BPS), - generally located 15-40 bp upstream the 3' splice site -, has probably disrupted essential sequences in the 3' end of the intron.

The results of RT-PCR from BHK cells transformed with PKD1 genomic sequences including the deleted intron, show that no normal transcript is produced by the deleted gene. This reduces the chances that this intronic mutation is leaky and that some normal product might be generated. However, A truncated polycystin-1 protein of 4188 instead of 4302 amino acids could be produced by the splicing mutation. This protein would lack the 96 C-terminal aminoacids, including part of the G-activation peptide, the PKA sites, the coiled coil domain and the SH3 domain consistent with the signaling function of the protein (Parnell et al, 1998) and the polycystin-2 interacting domain (Qian et al, 1997; Tsiokas et al, 1997). The resulting protein should be devoid of any possible channel regulatory function that polycystin-1 may have.

Previous studies have indicated no clear correlation between the severity of renal disease and the type or position of mutation. The two-hit process for cyst development (Qian et al, 1996; Wu et al, 1998) provides a ready explanation for the variability in PKD severity even within the same family. However, recently, significant interfamily differences have been related to different PKD1 mutations (Hateboer et al, 1999). Although our study provides only limited evidence, the intronic mutation here described, perhaps because found in a large pedegree, seems to be associated with a more severe form than ?? missense mutations. In the family with the deletion, three affected women died of cerebral ictus, one man died of proven subarachnoid haemorrhage, and a haemorrhagic ictus occurred in a 55 year old woman in dialysis; ESRD in two women occurred at 56 and 47 years. In the family with the L>P missense mutation, there are two affected women, one, 59 years old, with a middle renal insufficiency, and the second, 61 years old, with ESRD at 61. In the family with the R>Q missense mutation, one of the two affected women is 67 years old, with middle renal insufficiency. Because PKD1 gene is also expressed outside the kidney (Gabow, 1993), it is possible that specific PKD1 mutations behave differently in different cell types, playing a different role in the associated abnormalities. In addition, recent data in mouse revealed an important role for polycystin 1 in maintaining the structural integrity of the vasculature and suggest that the nature of PKD1 mutation contributes to the phenotypic variance in ADPKD (Kim et al, 2000).

In conclusion, we have identified 2 new missense mutations and 1 intronic mutation of the PKD1 gene. The missense mutations are located in the unique extracellular loop of PKD1 protein family. The intronic deletion involves the BPS and /or the pyrimidine rich region and leads to aberrant splicing.

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