SHORT REPORT

Mutation detection for exons 2 to 10 of the Polycystic Kidney Disease 1 (*PKD1*)-gene by DGGE

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The *PKD1*-gene encodes a 14 kb transcript spanning a 50 kb genomic interval. Two-thirds of the gene is reiterated at another locus on the same chromosome. Using Long Range PCR with primers in intron 1 and exon 11, 6.8 kb *PKD1* specific fragments were generated on genomic DNA. These products were used as templates for nested PCR's to screen exons 2–10 by Denaturing Gradient Gel Electrophoresis (DGGE). Upon analysis of 36 patients, a total of 11 different sequence variants were observed: A nonsense mutation in exon 2, a frameshift mutation in exon 8 and furthermore, two amino acid changes, two silent polymorphisms and five intronic variants.

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Introduction

Autosomal Dominant Polycystic Kidney Disease (ADPKD) has an incidence of 1 in 1000 in Caucasians. The formation of a large number of fluid-filled cysts in both kidneys together with interstitial fibrosis usually causes chronic renal failure in 50% of patients by the age of 60 years. Frequently occurring extra-renal manifestations are hypertension, cysts in liver and pancreas, and the occurrence of cerebral aneurysms. In 85% of ADPKD-families the disease is caused by a mutation in the *PKD1*-gene.¹ These patients have a more severe progression of the disease compared to patients with a mutation in the *PKD2*-gene.²

A large part of the *PKD1*-gene is reiterated at another locus on chromosome 16p, hampering mutation detection using straightforward techniques.³ The unique 3' part of the gene, exons 34–46, harbours only 10% of the mutations.⁴ Basically, the strategy to screen the reiterated part of the gene is to use long-range PCR with primers in unique parts of the gene, followed by nested PCR reactions

to amplify the different exons.^{5,6} We describe here the screening of exons 2–10 by DGGE in 12 overlapping fragments.

Methods

Patients

In this study we analysed DNA from 36 individuals diagnosed as having ADPKD, 25 linked to the *PKD1*-gene on chromosome 16, other families are too small to establish linkage. The majority of patients have been analysed for most of exons 15 and 23–46.

PCR amplification of DNA

LR-PCR was performed, in 50 µl reactions, using the EXPAND^{TM} PCR system (Roche, Mannheim, Germany) with buffer system 3 containing: 2.25 mM MgCl₂, 50 µM of each dNTP, 300 nM of each primer (pPKDin1AF and pTr) and 250 ng genomic DNA. Samples were amplified in 0.2 ml thin-walled tubes using a Perkin Elmer DNA thermocycler (Geneamp PCRsys 2400, PE Applied Biosystems, Nieuwerkerk a/d/ IJssel, The Netherlands). The PCR programme recommended by the manufacturer was used with denaturing, annealing and elongation temperatures of 94, 66, and 68°C respectively and elongation times of 10 min increasing with 20 s per cycle.

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Nested PCR's for exons 2–10 were performed on the 6.8 kb pPKDin1aF-pTr products isolated from gel using low melting-point (LMP) agarose. Samples were always loaded with empty lanes in between. Nested PCR's were performed on 10⁵ times diluted products with intronic primers to amplify all exons and adjacent splice and branch sites. Primers were selected using the melt map programme WINMELT version 2.0. For each fragment a 50 or 55 bp GC-clamp was attached at the 5' or 3' end of one of the primers.

A single set of primers was used for each exon except for exons 5 and 10 where two and three pairs were needed respectively (Table 1).

Denaturing gradient gel electrophoresis (DGGE)

Amplified fragments were analysed on a 9% polyacrylamide gel (37.5 : 1; Mallinckrodt-Baker, Deventer, The Netherlands) with linear denaturing gradients of 40 to 100% of a mixture of 40% formamide (Mallinckrodt-Baker) and 7 M urea (USB, Amersham Pharmacia Biotech, Roosendaal, The Netherlands), for details see Table 1. Electrophoresis was performed at a constant temperature of usually 58°C, at 100 V for 8–16 h.

Results

Alignment of the *PKD1* genomic sequence and the sequence of a Bacterial Artificial Chromosome (BAC) clone that

contains two copies of the homologous loci (Genbank AC002039), was previously shown to reveal regions in the *PKD1*-gene that were not duplicated in the two homologous genes.⁷ In one of these regions in intron 1, close to exon 2, we selected a PCR primer for long-range PCR (pPKDin1aF). The other primer (pTr) is located in exon 11. To validate specificity of the amplified products radiation hybrid HJ145.19, which contains only the *PKD1*-gene and the rodent-human somatic cell hybrid N23HA, which contains only the homologous region, were used as controls.³ Long-range PCR revealed in human DNA a 6.8 kb band that was also present in HJ145.19 but absent in N23HA (Figure 1). In addition a 1.2 kb band was observed sometimes in N23HA, although with varying intensities. This band was always absent in HJ145.19, indicating that it may be a product of one of the copies of the PKD1 gene at a proximal location on chromosome 16 (Figure 1). We therefore always isolated the 6.8 kb band from a low melting point agarose gel, and nested PCR's were performed on a 10⁵ dilution of this product. Nested PCR's never revealed products on the 6.8 kb region isolated from gel in the N23HA control somatic cell hybrid (not shown).

Denaturing gradient gel electrophoresis for *PKD1* exons 2 to 10

For DGGE-analysis gene-specific fragments of 119–372 bp were generated. A single set of primers was used for each exon

Table 1 Primers and DGGE conditions

Primer name	Sequence (5'–3')	Size (bp)	Tann. (°C)	DGGE conditions
ex2F	^b AGGCCATAGCTTGGGGATGC	193	60	9%PAA 40%–100%UF 58°C
ex2R	CACGACTGGGGGACACTCAC			
ex3F	GGCTCCTGGCGAACCTCTCG	262	55	6%PAA 40%–70%UF 58°C
ex3R	^b TCCAGCCAGGACCCACCCA			
ex4F	^a GGCATAGACCTTCCCACCAGA	340	60	9%PAA 40%–100%UF 58°C
ex4R	ACTCACAGGCTCCCATGCT			
ex5aF	^a AGCATGGGAGCCTGTGAGT	372	55	9%PAA 40%–100%UF 58°C
ex5aR	GGAAGACGTGCTGGAGGAG			
ex5bF	^a GCCCTCCAGTGCTCTCTT	352	55	9%PAA 40%–100%UF 58°C
ex5bR	CTTCCACCTGCACGCTCTGT			
ex5cF	CTCGCATCGCTATGTGCTGC	337	60	9%PAA 40%–100%UF 58°C
ex5cR	^b AGGGTGTCAACGGTCAGTG			
ex6F	GCAGAGCCTGGTACCCCGT	346	55	9%PAA 40%–100%UF 58°C
ex6R	^c ACCTCCTTCCTCCTGAGACT			
ex7F	GGAAAGGGGACAGTGATGGG	395	60	9%PAA 40%–100%UF 58°C
ex7R	CCAGGCTCCACCGCGGGCGC			
ex8F	CGGTCCCCAGTCTGTTCTGTC	293	55	9%PAA 40%–100%UF 58°C
ex8R	^b AACATTAAGCCCCCAAG			
ex9F	^b TTCGGGTAGGGGAGTCTGG	281	60	6%PAA 40%–100%UF 58°C
ex9R	GGACCAGCAGACGTGAAAGC			
ex10aF	^b GCAGTTGGGCATCTCTGA	291	55	9%PAA 40%–100%UF 58°C
ex10aR	CGGGAACGGAGAAGAGGA			
ex10bF	TCTGCTTGCCGCTGGACGCC	119	64	9%PAA 40%–100%UF 58°C
ex10bR	^b GGGAACGGAGAAGAGGAA			
PKDint1af	GGG GAG GTG GAG TTC TCG GTT TGC C			
pTr	CCT CCA AGT AGT TGC GCT GTG ATC G			

^a1 50 gc-clamp (5'–3'): CGCCCGCCGCGCCCGCGCCCGCGCCCGCCCGCCCGCGCCCGCCG. ^b2 55 gc-clamp (5'–3'): GCCCGCCGCGCCCGCGC–CCCGCCCGCCCGCCCGCGCCCGCGCCCGCGCCCGCGCCCGCGCCCGCGCCCGCCG. ^c3 60 gc-clamp (5'–3'): CGCCCGCCGCGCCCGCGCCCGCGCCCGCGCCCGCGCCCGCGCCCGCGCCCGCCG.

Figure 1 Long Range PCR. LR-PCR for exon 2–10, with primers PKDint1aF in intron 1 (position 17621) and pTr (position 24449) in exon 11, generates a 6.8 kb fragment for the *PKD1*-gene and a 1.2 kb fragment for a homologous locus (HG). (1) Hybrid cell line N23HA, contains the homologous loci (HG) and not the *PKD1*-gene. (2) Hybrid cell line HJ145-19, contains the *PKD1*-gene and not the HG-loci.

except for exons 5 and 10 where two and three pairs were needed respectively. A total of 11 different sequence variants were observed (Table 2) that were confirmed on fresh LR-PCR's followed by nested PCR's on DNA of the patients and/or additional family members.

Truncating mutations Analysis of exon 5 (fragment 5C) revealed a nonsense mutation in family PK4. This mutation, R400X predicts a translation stop at amino acid 400 and generates a *DdeI* restriction site. The index patient of this family died at 68 years of age from cardiac infarction. Polycystic kidneys were an unexpected finding at autopsy. In total 15 affected family members were collected, two of them reached end stage renal failure (ESRF) before 50 years.

Analysis of exon 8 revealed a frame shift mutation in family PK10. Deletion of a C in a stretch of 4C's at position 1703–1706, predicts a frame shift at amino acid 596 resulting in a truncated protein of 610 amino acids. The index patient was a male, with polycystic kidney and liver disease, who entered into renal failure at 69 years and died at the age of 72 due to heart failure. He had six children with polycystic kidney disease. One of them entered into renal failure at the age of 31.

Coding and non-coding polymorphisms Two amino acid substitutions were identified: 276G>A in exon 2, resulting in the amino acid substitution A92T⁴ was once identified. 1919C>T, in the overlap of fragments ex10A and ex10B, results in the substitution A637V and was identified in four families including family 10 for which we identified a frame shift mutation in exon 8 (see above). Analysis of exon 5

Table 2 Mutations and polymorphisms. Mutations and polymorphisms were designated according to the recommendations of Antonorakis *et al* ⁽¹³⁾ using the *PKD1* sequence with Genbank accession number U24498

Mutations:

Nucleotide change	Amino acid change	Exon	Patient
1998C>T	R400X	5	PK4
1706delC	fs596 (stop at 610)	8	PK10

Polymorphisms:

		Exon	Frequency
276G>A	A92T	2	1/72
1910C>T	A637V	10	4/72
1023C>T	A341A	5	2/72
1120T>A	L373L	5	1/72
Intron			
216-79C>T	-	1	2/72
1620-39C>T	-	7	1/72
1722+23C>T	-	8	1/72
1723-27G>A	-	9	11/72
1850-4G>T	-	9	6/72

revealed two silent mutations 1023C>A (A341A) and 1120T>A (L373L) both also described by Rosetti *et al*.⁴ Five intronic polymorphisms in introns 1 (216–79C>T), 7 (1620–39C>T), 8 (1722+23C>T and 1723–27G>A) and 9 (1850–4G>T) were observed with varying frequencies.

Discussion

We have performed mutation detection for the *PKD1*-gene to gain insight into the mutation spectrum but also for diagnostic applications in ADPKD patients. Sequencing the entire 14 kilobases of coding DNA of the *PKD1* gene is a daunting task. We therefore prefer to pre-screen fragments of the gene searching for variants. We have chosen to screen the *PKD1*-gene by DGGE because the sensitivity to detect sequence variants can reach to a value close to 100% when a GC-clamp is introduced in the fragment during PCR.⁸ Exons 2–10 comprise approximately 1.7 kb (13%) of coding sequence in which we identified two (5%) truncating mutations in 36 patients and several variants were identified which are likely to be non-disease causing polymorphisms. These data are in a similar range as observed by Rosetti *et al*.⁴ who screened the same region of the gene by direct sequencing. However, Phakdeekitharoen *et al*.⁹ did not find any mutation in this part of the gene by heteroduplex analysis in 40 Thai families. DGGE-analysis for other parts of the gene have been reported previously.^{10,11}

All the available data suggest that with a few exceptions⁴ each family with ADPKD has its own 'private' mutation. The mutation detection rate for the gene as a whole is around 60%

indicating that promotor mutations, missense mutations, deletions or variants with unknown effects on splicing may also be responsible for the disease. So far the mutations appear to be spread evenly over the entire *PKD1*^{4,11} and also *PKD2*¹² gene with no clustering indicative of mutation hot spots.

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