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LETTER TO JMG

Mutation screening of the *PKD1* transcript by RT-PCR

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Autosomal dominant polycystic kidney disease is a common disorder, primarily characterised by progressive renal enlargement and cyst formation, leading, in the majority of patients, to end stage renal disease. Cyst formation has also been observed in liver and pancreas. Cerebral artery aneurysm has also been reported.[1]

This disorder is genetically heterogeneous, but about 85% of cases are the result of a mutation in the *PKD1* gene, located on the short arm of chromosome 16 (16p13.3). This gene spans about 50 kb of genomic DNA, is composed of 46 exons, and the ORF is 13 kb.[2–5]

The incidence of *PKD1* in the population is about 1/1000 subjects, making it the most common genetic disorder in humans.[6] However, routine clinical diagnosis by molecular analysis has not been extensively developed. This is because of two main characteristics of the gene. Firstly, the coding region is large and spans 46 exons. The second and main reason is that sequences homologous to 70% of the coding region are present as nearly identical transcribed copies located closer to the centromere on the short arm of chromosome 16.[7 8] Some of these sequences are transcribed.[2] This situation has led, until recently, to identification of mutations in only 30% of the non-repeated part of the coding region. Previously, authors have reported RT-PCR based mutation detection, but they only screened the *PKD1* gene from exon 16 to exon 46. Recently, two papers have reported different screening strategies for mutation detection in the repeated region of *PKD1*.[9–11] One involves a long range RT-PCR approach (*PKD1* is expressed in lymphocytes) designed to amplify a 13 kb RT-PCR product.[12] This can probably not be used as a routine screening test in a hospital setting. The second approach involves PTT tests and sequencing of genomic DNA.[13] This approach is more practical and has met with some success, although the detection of mutations is mainly based on protein truncation tests, which allows the detection of premature protein translation.

The molecular diagnosis of this disorder is, however, important not only for patients but also for mutation based selection of patients for therapeutic trials in the future. We therefore designed primers from database sequences, spanning regions in which we detected nucleotide differences between *PKD1* and its homologues. We used these primers to amplify RT-PCR products, ranging from 750 bp to 2.3 kb, a fairly normal range, and have shown that they are *PKD1* specific. We used this test to screen for mutations in nine polycystic patients, with a clinical presentation of *PKD1*, with or without a family history. Surprisingly, we found 17 polymorphisms and six mutations. In conclusion, we have developed a strategy for mutation screening over the entire *PKD1* coding region by a quick and reliable method.

MATERIAL AND METHODS

Patients

This study comprised nine patients with autosomal dominant polycystic kidney disease (ADPKD). Subjects gave informed

Table 1 Sequences and characteristics of PKD1 specific primer pairs. (positions where differences between PKD1 and PKD1 related sequences have been reported are underlined)

Primer pair	Sequence: 5′-3′	Position in cDNA	Amplicon size (bp)	Annealing temp (°C)
1F	CTCAGCAGCAGGTCGCGGCC	76	856	66
1R	AGGCACTGGAGGGCTGGGCCGC	932		
2F	CAGGGCTGGTGCCTGTGTGGGGCG	890	1313	72
2R	GGCCTGGGGGTGGCAAGAGGCGTC	2203		
3F	GGACGCCTCTTGCCACCCCC	2179	2312	70
3R	CTGGCACGGGTGGGGGCGGCTTCC	4491		
4F	AAGCCGCCCCCACCCGTGCCA	4470	750	65
4R	CTCCAGGCAGTCCAGCTGTAGGAGAC	5220		
5F	GGGATGGCACCAACGTCTC	5181	1541	63
5R	CCTCCAAGTAGTTGCGCTGTGATCGC	6722		
6F	AGCGCAACTACTTGGAGGCCC	6705	2202	62
6R	ACCACAACGGAGTTGGCGG	8907		
7F	CAGCTCCGCCAACTCCGCCAACT	8875	2106	72
7R	GGACAGGAGCCACGCAACACTCAC	10 981		
8F	AGCCAGCGAGGCTGTCCAGG	10 803	1818	65
8R	GCAGCCTGCGCAGGAACAACTCC	12 620		
9F	CCTCTCGCTGCCTCTGCTCACCTC	11 896	1795	68
9R	ATAACGCCACCACACCTACCAAGC	13 691		

Table 2 PCR primers for SSCP

Fragment	Primer	Sequence	Annealing T
Fragment 1			
1	AB1F	GCCATGCGCGCGCTGCCCTAACG	60°C
	SAB1B1	GAGGTTCGCCAGGAGCCCAACG	
2	SAB1F2	ACA GCG CTA GAC GTC TCC C	60°C
	SAB1B2	AAG GGG ATG CCA AGC AGA GG	
3	SAB1F3	CCA CGT GTG CTG GGC CTG G	60°C
	AB5B	GGCACTGGAGGGCTGGGCCGC	
Fragment 2			
1	AB5F	CAGGGCTGGTGCCTGTGTGGGGCG	60°C
	SAB5B1	CACATAGCGATGCGAGGCAGCCGG	
2	SAB5F2	GCT CCG CCG AGG TGG ATG CC	62°C
	SAB5B2	TGT CCG AGG GGC AGA GCG GG	
3	SAB5F3	TGG TTC AGG CCT GGA GGC	56°C
	SAB5B3	TGG CAG CTC TCC AGG CTG	
4	SAB5F4	TCA CCA GGA GCC TAG ACG	52°C
	SAB5B4	AAT ACC ATG ACC TCC ACG	
5	SAB5F5	GCA GGA CGG CCT CTC AGC CCC	60°C
	AB10B	GGCCTGGGGGTGGCAAGAGGCGTC	
Fragment 3			
1	AB10F	GGA CGC CTC TTG CCA CCC CC	60°C
	SAB10B1	GCT GGC AAG TGG GGC AGC CA	
2	SAB10F2	TCT CCG CCA ACG CCT CGT CA	60°C
	SAB10B2	GTG GCG TTG GCA CCA GAG TC	
3	SAB10F3	CAG CCT TGG TGC TCC AGG TG	64°C
	SAB10B3	GAC TCC CTG CAG TAC ACG GG	
4	SAB10F4	GAG CCC ATC TGT GGC CTC C	60°C
	SAB10B4	GTG GAG ACC TGC AGA CCC TG	
5	SAB10F5	CGT GAA CTA CAA CGT AAC CG	62°C
	SAB10B5	GCA GCG TAG GTG TGC ATG AC	
6	SAB10F6	GGT GCT GGT GGA GCA CAA TG	60°C
	SAB10B6	CGA GGC ATA GGT GTG GTT GG	
7	SAB10F7	CTG TCC TGA CCC AGA GCC	58°C
	SAB10B7	GCA GGT ACA CAT GCT CCA C	
8	SAB10F8	GTG GAC CTT CGA CAT GGG	58°C
	SAB10B8	CAC GGT CGT GTT GGA GGA G	
9	SAB10F9	CCTCTTCGACTGGACCTTCGGGG	60°C
	AB15B1	CTGGCACGGGTGGGGGCGGCTTCC	
Fragment 5			
1	AB15F2	GGGATGGCACCAACGTCTC	62°C
	SAB15B1	CCT CCT CCA AGG ACC AAG TG	
2	SAB15F2	CTG GTGGCA GTG GTG TCG	60°C
	SAB15B2	CAG CAC CAG CTC ACA TTG G	
3	SAB15F3	TGC CCT TTT GGG GGC AGC	63°C
	SAB15B3	GGC GGA AGG TGA CAG CTG AGC	
4	SAB15F4	CAG ATC CTG CTG GCT GCC G	65°C
	SAB15B4	GTA GGC GAC CCG AGA GCC GC	
5	SAB15F5	TTC ACA GCC CGC GTG CAG C	63°C
	SAB15B5	GGT GGC GGC CTC AAA CTG C	
6	SAB15F6	CTG CTT CAC CAA CCG CTC GG	65°C
	30Ext	CCTCCAAGTAGTTGCGCTGTGATCG	
Fragment 6			
1	FQF26	AGC GCA ACT ACT TGG AGG CCC	62°C
	SAB26B1	ATG AGC CAC CCT CAA TGA TGG	
2	SAB26F2	CAG GCC AAT GTG ACG GTG G	60°C
	SAB26B2	CCT TCC ACA CGG TCA GGC TG	
3	SAB26F3	CGG CTG GCG TGG AGT ACA CC	60°C
	SAB26B3	CAG TCG CAT GCC TGC ACT GC	
4	SAB26F4	TGG ATG AGA CCA CCA CAT CC	62°C
	SAB26B4	CAG CAG CAG GGC GTA CAC C	
5	SAB26F5	CTT CGA ATG CAC GGG CTG G	62°C
	SAB26B5	GTG CAG CCA GAC TGT GAG CC	
6	SAB26F6	TCA ACA GGT CTT TGG CCA TC	62°C
	SAB26B6	CGA TCT GCT GGA TGT CAT CC	
7	SAB26F7	GAC TCT GGT GTC CCT GAG GG	60°C
	SAB26B7	CAT CCG AGA TGG TGA CTC G	
8	SAB26F8	GCC AGC TCG GAC GTG CGG	60°C
	SAB26B8	GTC ACT GAG GTT GGC CAG G	
9	SAB26F9	CCA CTT CTC CAT CCC CGA GG	62°C
	AB21B	ACC ACA ACG GAG TTG GCG G	
Fragment 7			
1	AB21F	CAGCTCCGCCAACTCCGCCAACT	62°C
	SAB21B1	GCT GGG TCT CTG CTC CCC GG	
2	SAB21F2	CTG ACC ACC GGC CCT ACA CC	62°C
	SAB21B2	AGC GGA CAT GGC TTG GGG C	
3	SAB21F3	CCT GGA GGA GAC CTC GCC	62°C
	SAB21B3	GTG GCC GCT CCG GCT GTC CAC C	
4	SAB21F4	ATC CTC GTC AAG ACA GGC	54°C
	SAB21B4	GAA AGC CAG TCA TTG ACC	

contd

Table 2 *continued*

Fragment	Primer	Sequence	Annealing T
5	SAB21F5	GAC CTG CAG ACG GCA CGC	64°C
	SAB21B5	AGT CGC CAA CAG CCC CG	
6	SAB21F6	CTG CCT CTT CCT GGG CGC C	66°C
	SAB21B6	GCG TGG AGG CCT GAG AAC G	
7	SAB21F7	TGG ACT CGT CCG TGC TGG	62°C
	SAB21B7	GGA TCA GGT CTT CAT CTG ATG C	
8	SAB21F8	CCC CTA CTC GCC TGC CAA ATC C	68°C
	SAB21B8	TCC TGG ACA GCC TCG CTG CC	
9	SAB21F9	GAC GCT GGC GCT GCA GAG GC	68°C
	AB36B	GGACAGGAGCCACGCAACACTCAC	
Fragment 9			
1	AB42F	CCTCTCGCTGCCTCTGCTCACCTC	62°C
	SAB42B1	AGC GAG GCC GCC AGG CCA CG	
2	SAB42F2	CCA GGT GGC GCA GCT GAG CTC	62°C
	SAB42B2	CAG GAC TCG GCA GGA CAC AGG	
3	SAB42F3	TGG TGC TGT GCC CTG GGA CTG G	62°C
	SAB42B3	GAG GGC AGC GGC TCC ATC CCT TC	
4	SAB42F4	GGA GT CCG CCA CAA AGT CC	64°C
	SAB42B4	CCT TGC AGG CTG TGC AGC	
5	SAB42F5	CAA CCA GGC CAC AGA GGA CG	62°C
	SAB42B5	CTC GGC CTT GAC AGC GGC	
6	SAB42F6	AGT CGG AGT GGA CAC CGC	62°C
	SAB42B6	CAT CTG CCC AGG GGG TGG	
7	SAB42F7	CTC AGG TAC AGC GGG CTG TGC	62°C
	AB46B	ATAACGCCACCACACCTACCAAGC	

consent and the hospital ethics committee approved the study. All patients, except patient 4, had a familial history of ADPKD. The patients under 30 years old all had at least two cysts in one kidney or one cyst in each kidney, those aged between 30 and 59 had at least two cysts in each kidney, while those aged 60 or older had at least four cysts in each kidney. The parents of patient 4 had no kidney cysts at an age greater than 60.

Eight patients (1, 2, 3, 4, 5, 6, 7, and 11) had end stage renal disease. Seven (1, 2, 3, 4, 5, 6, and 7) were undergoing haemodialysis treatment and patient 9 had received a transplant. Patient 8 had normal renal function. Families 1, 6, 7, and 8 had a history of cerebral aneurysms.

The polycystic kidney disease segregates over at least three generations in eight of the families. Patient 4, however, had no familial history and is a sporadic case. However, this patient fulfilled all the *PKD1* criteria and his clinical presentation was identical to that of the familial cases. The presence of another nephropathy segregating in the families was excluded.

Seven of the families are of European origin, family 5 is from North Africa, and family 6 is from the Middle East. No linkage analysis was performed because some members of the families refused to contribute to the study.

RT-PCR analysis of *PKD1*

Using Blast2 software, we compared the sequence of *PKD1* (accession number L33243) with the sequence of a BAC containing *PKD1* homologues (accession number AC002039) and detected the regions with the lowest nucleotide identity. We used these data to design a set of nine primer pairs specific for the *PKD1* transcript, which encompass the whole coding region. A summary of these primers is presented in table 1.

Lymphocytes were isolated using a Ficoll gradient and total RNA was extracted using the Rneasy Quiagen kit, according to the manufacturer's instructions (Quiagen SA). Total RNA was resuspended in Rnase free water. A total of 1-5 µg of total RNA was reverse transcribed using a RT-PCR kit (RT Life Technology GIBCO BRL), according to the manufacturer's instructions, and 3 µl of the RT products were amplified using a standard hot start PCR technique using the primers; the PCR temperature is shown in table 1. $MgCl_2$ concentration in PCR reactions was 1.5 mmol and 35 cycles of amplification were performed.

Mutation screening

RT-PCR products, synthesised as previously described, were diluted to 1/1000 and subjected to a nested PCR, using internal

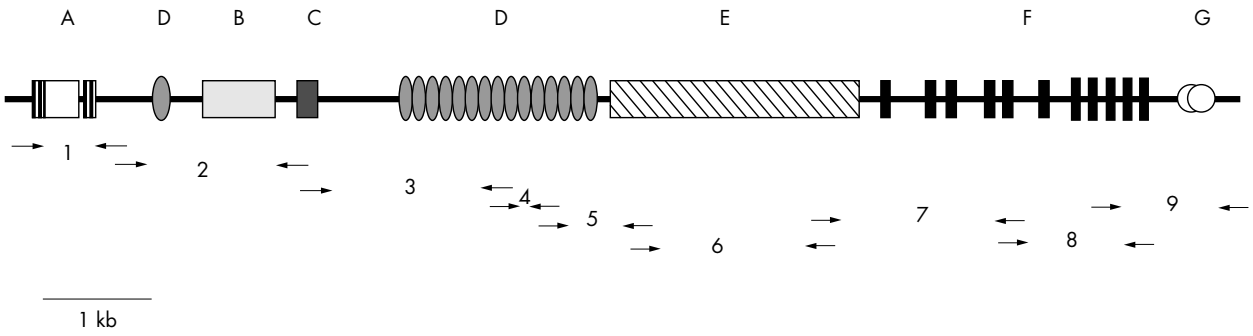

Figure 1 Distribution of primers along the *PKD1* transcript. Protein domains are lettered from A to G. A: leucine rich repeats. B: lectin C domain. C: LDL-A domain. D: PKD domain. E: REJ domain. F: transmembrane domains. G: coil-coiled domain. Primer sets are numbered underneath.

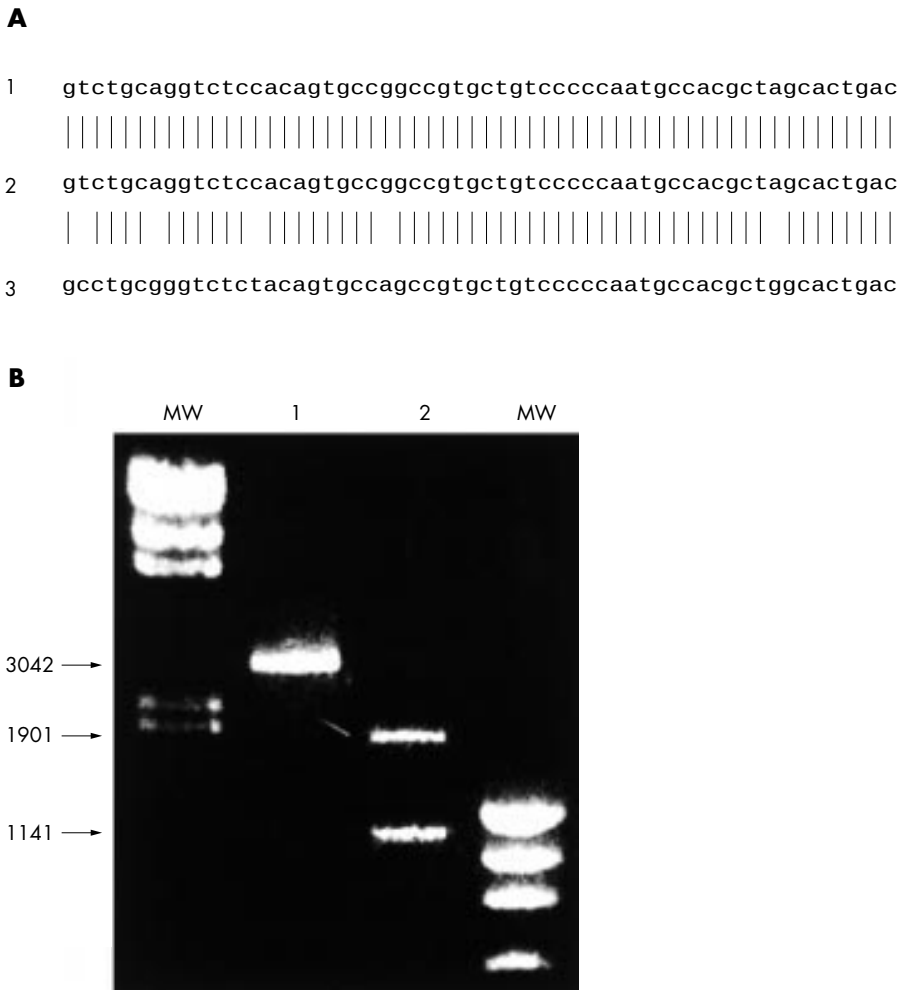

Figure 2 (A) Comparison of the sequences of *PKD1* (1) (nt 3273 to 3330 of the cDNA sequence) to the sequence we obtained using our RT-PCR test (2) and the sequence of one homologue of *PKD1* (3). (B) Enzymatic cleavage of a PKD1 specific RT-PCR product (fragment 3) with the restriction enzyme *Nhe*I, which cleaves the *PKD1* sequence (nt 3322 of the cDNA sequence), but not the sequence of homologues. Lane 1: undigested RT-PCR product. Lane 2: digested product. MW: molecular weight markers.

primers derived from the *PKD1* sequence, in order to generate fragments of about 200-300 bp (table 2), except for PCR products corresponding to fragments 4 and 8, which were directly sequenced. A total of 40 µl of PCR product was mixed with 40 µl of 0.1 mol/l NaOH and transferred to ice. This mixture was deposited at the top of a 30% acrylamide gel and subjected to electrophoresis (16 hours at 10 mA at 18°C). If necessary, a second analysis was performed at 4°C. Following migration, the gel was stained with ethidium bromide and fragments were visualised by UV. Fragments (PCR products) showing an abnormal migration compared to standard DNA from unaffected subjects were sequenced on both strands.

Sequences were analysed and compared to the *PKD1* gene sequence (accession number L33243) and homologous sequences (accession numbers AC002039, AC009065,[8] AC012171,[8] NT_024838, and NT_010543), using the basic Blast software (http://www.ncbi.nlm.nih.gov/blast/).

RESULTS

Designing an RT-PCR assay specific for the *PKD1* transcript

Using database sequences (see Methods), we compared the sequence of *PKD1* to sequences of *PKD1* homologous transcripts.[7] The comparison of these sequences was used to design a set of nine oligonucleotide primer pairs (table 1), for which at least the 3′ end is specific for *PKD1*. The distribution of these primers is shown in fig 1 and covers the entire coding sequence.

We then extracted RNA from normal lymphocytes and reverse transcribed it using a standard procedure. We amplified the cDNA using the primers described above. The amplified products were directly sequenced and compared to *PKD1* and related sequences.[7 8] Sequencing of DNA fragments obtained by our RT-PCR assay clearly corresponds to unique sequences, corresponding to *PKD1* only and not to any of the related sequences (an example is shown in fig 2A). Moreover, we used enzymes that specifically digest the *PKD1* sequence and not the related sequences, and showed that each amplified fragment is specific for *PKD1*. An example is presented in fig 2B.

These data clearly indicate that the RT-PCR assay described above amplifies the *PKD1* transcript only and not transcripts from related sequences.

Mutation screening of *PKD1* using a specific RT-PCR assay

In order to test the ability of our RT-PCR assay to identify mutations in ADPKD patients, we selected nine unrelated patients who presented the classical symptoms of the disorder. RNA was extracted from fresh blood lymphocytes and RT-PCR was performed. Before screening for mutations, we ruled out the possibility that the transcript corresponding to the mutant allele was unstable and degraded in the majority of our

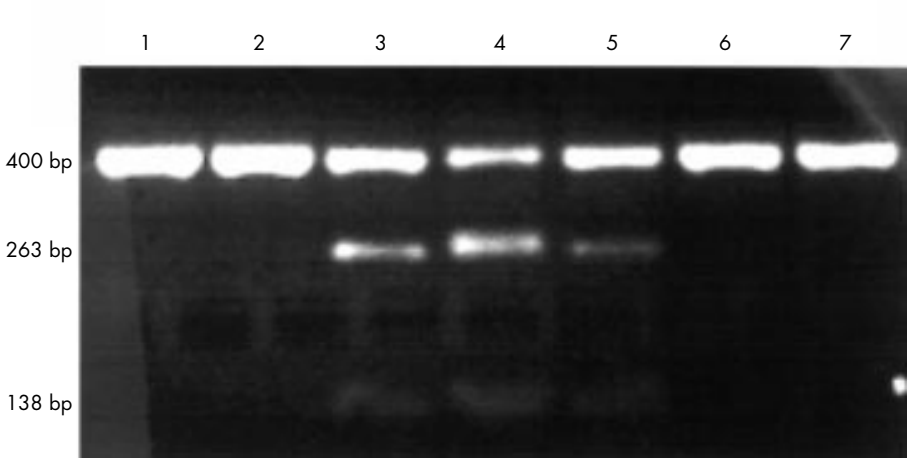

Figure 3 Test of the expression of the two *PKD1* alleles in patients 1-7. A *PKD1* specific RT-PCR fragment was digested with the restriction enzyme *Tse*I, which specifically digests the *PKD1* polymorphic allele (A12273G). This clearly shows that two alleles of *PKD1* were amplified in patients 3, 4, and 5.

patients, as this would have rendered our assay ineffective for *PKD1* mutation screening. In order to do this, we used known *PKD1* polymorphisms and digested the RT-PCR product with the appropriate restriction enzymes. An example is given in fig 3. We can see that two alleles can be detected in the RNA of ADPKD patients. Moreover, we found polymorphisms in all tested patients (see below) except one (patient 1), indicating that the two alleles have been amplified in the RT-PCR reaction.

As a next step, we screened for mutations in RT-PCR products, using a standard SSCP approach, followed by sequencing (fig 4A, B). Surprisingly, we found 17 differences from the published sequence (table 3). These differences clearly represent polymorphisms in the repeated region, since the variation in the sequence is present in several patients, as well as in unaffected subjects, some of which have already been published. It can be seen in table 2 that 13 mutations involve the base combination of C/T (or G/A), while only four affect other base combinations (75%/25%). Moreover, of the missense mutations, seven of 17 polymorphisms (six already reported and one new one) correspond to a substitution present in at least one of the *PKD1* homologues (40%).

In addition, we detected six mutations which were each present in only one patient and were not detected in unaffected subjects. One is a 4 bp insertion (patient 6), which has not been previously described (2986ins4). This mutation introduces a stop codon 300 bp downstream. The second is a splice mutation (patient 1) that causes partial deletion (74 bp) of exon 14 and introduces a stop codon 400 bp downstream, which has already been described.[12] This splicing abnormality is caused by a mutation in the splice acceptor site of exon 14 and the use of a cryptic splice site in exon 14. The third is a splice site mutation that causes exon 44 to be skipped, thus deleting 45 amino acids coding for the tenth transmembrane

Table 3 Polymorphisms and mutations found in nine unrelated patients

Position in the cDNA	Position in the protein	Already described	Present in patients (No)	Present in homologues
Polymorphisms				
A759G	P253P	No	Control	Yes
T1119C	L373L	Yes[8]	6 and 8	Yes
A2214G	Q738R	No	5	No
G2700A	P900P	Yes[14 15]	4	No
C2730T	D910D	Yes[14 15]	4	No
T3063C	G1021G	Yes[14 15]	3	Yes
T4195C	W1399R	Yes[14]	3	Yes
C4335A	V1445V	No	4	No
A4665C	A1555A	Yes[14 15]	3, 4, 5	Yes
C6299T	S2029L	No	7	No
T7165C	L2389L	Yes[15–17]	3, 4, 5	Yes
C7441T	L2472L	Yes[15]	3, 4	No
T7708C	L2570L	Yes[15]	3	Yes
G8545C	A2848A	No	8	No
A12130G	I4044V	Yes[13 18–20 22]	3, 4, 5	No
IVS 44+19 delG		Yes[13 18 19 22]	6	No
A12273G	A4091A	Yes[13 18 19]	3, 4, 5	No
Mutations				
IVS13-2 A>T	Frameshift L1054fsx1074	Yes[12]	1	No
IVS 44 +2 T>C	Del 4001-4045. 4001del46	No	4	No
IVS 45 -14 T>C	Insertion 30 AA 4148-4149ins30	No	5	No
2986 Ins 4bp	Frameshift (1099) S995fsx1099	No	6	No
G3961T	Q987H	No	7	No
9412del3	3138 delV	No	11	No

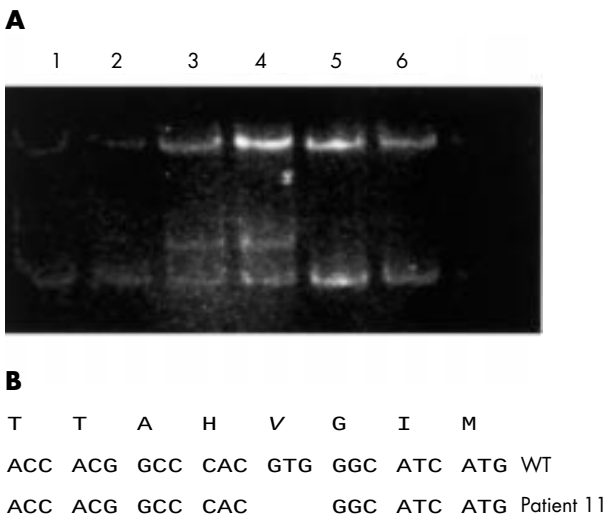

Figure 4 Mutation screening in patients. (A) SSCP analysis of patient 2 (lane 1), patient 3 (lane 2), patient 11 (lane 3), his affected daughter (lane 4), patient 4 (lane 5), and patient 5 (lane 6). (B) Sequencing of the abnormal fragment of patient 9 showed a 3 bp deletion, removing a conserved amino acid (V) of the PLAT domain.

domain.[13] The sequence of the corresponding region of this patient's genomic DNA showed a T>C transition at the intron 44 donor site (fig 5). This mutation was detected in patient 4, a sporadic case of PKD with no previous familial history. The fourth mutation (patient 5) is also a splicing mutation that leads to intron 45 being unspliced, adding 30 extra amino acids to the protein. Sequencing exons 45 and 46 and intron 45 only showed a T>C transition in the intron (51385, IVS45-14, fig 6). These three splicing variants were detected by RT-PCR products of an abnormal size (for example, see fig 5). Abnormal fragments were sequenced, as well as the corresponding genomic region. The fifth mutation (patient 9) corresponds to a 3 bp deletion (9412-9415), encoding a valine (3138) which has been strongly conserved throughout evolution (human, mouse, Fugu). This valine is part of a PLAT domain, which is involved in signal transduction.[21] This mutation segregates with polycystic kidney disease in this family. The sixth mutation (patient 7) is a missense mutation (Q987H) that has not been previously identified. The absence of these mutations in 100 unrelated wild type chromosomes has been verified. This mutation is likely to be pathogenic as it changes a negatively charged amino acid (glutamic acid) into a positively charged one (histidine). Moreover, the mutated glutamic acid is conserved in *PKD1* from human to Fugu.

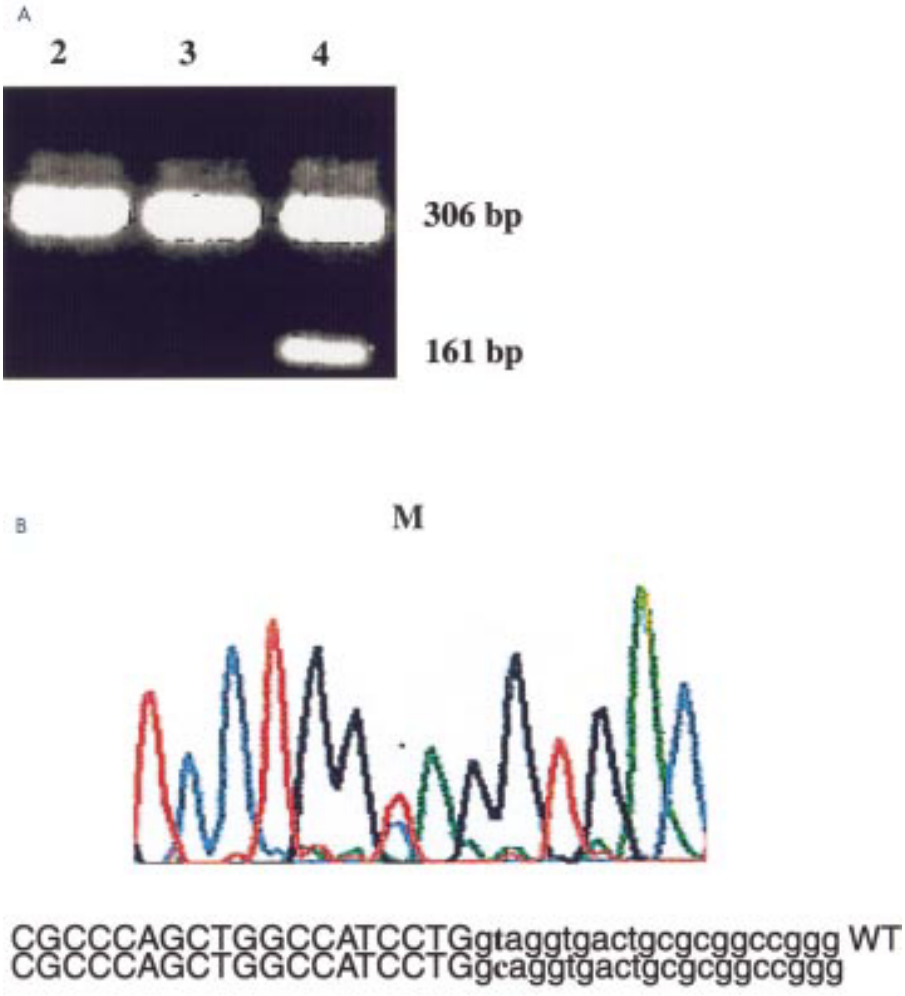

Figure 5 Exon skipping in patient 4. (A) RT-PCR reaction, spanning exons 43 to 45, was performed on cDNA from patient 2 (lane 2), patient 3 (lane 3), and patient 4 (lane 4). Products were analysed on 1.5% agarose gels. Sequencing of the abnormal 161 bp fragment showed skipping of exon 44 in patient 4 *PKD1* transcript. (B) Sequence of the genomic region spanning the boundary between exon 44 and intron 44. A T-C transition, affecting intron 44 donor site (GT to GC) can be seen.

GGAGAGCTGTACCGGCCGGCCTGGGAGCCCCAGGACTACGAGATGGTGGAGTTGTTCCTGCGC
G E L Y R P A W E P Q D Y E M V E L F L R

AGGCTGCGCCTCTGGATGGGCCTCAGCAAGGTCAAGGAGgtgggtacggcccagtgggggggga
R L R L W M G L S K V K E V G T A Q W G G

gagggacacgccctgggctctgcccagggtgcagccggactgactgagcccc**t**gtgccgcccc
E G H A L G S A Q G A A G L T E P L C R P

cag**TTCCGCCACAAAGTCCGCTTTGAAGGGATGGAGCCGCTGCCCTCT**
Q F R H K V R F E G M E P L P S

Figure 6 Splicing anomalies in patient 5. Genomic sequence from exon 45 to exon 46 of *PKD1*. Sequence variation in intron 45 of patient 5 (T to C, nucleotide 51 385) is indicated in bold.

DISCUSSION

Here we describe a *PKD1* mutation screening strategy based on RT-PCR amplification of the transcript, using specific primers. In order specifically to amplify the *PKD1* transcript, and not any of the homologous sequences, nine sets of primers specific to the *PKD1* sequences were designed on the basis of comparisons with *PKD1* homologues present in the databases. In order to check the specificity of this assay, we sequenced RT-PCR products. The sequence of these fragments corresponds to a unique sequence, which is the sequence of *PKD1* and not one of its six homologues. We cleaved the RT-PCR product with selected restriction enzymes, allowing the discrimination of *PKD1* from related sequences and confirming that the sequence is definitely the *PKD1* sequence.

Using RT-PCR, we amplified the *PKD1* transcript from nine unrelated patients and checked that the two alleles could be amplified and analysed, using a published polymorphism.[22] This is extremely important, as mutations can often lead to unstable transcripts that cannot be detected. Three patients out of nine show the two polymorphic alleles, indicating that the transcripts from the two alleles can be amplified. Moreover, we have detected polymorphisms by SSCP in all patients except one. This indicates that we can screen both copies, even if one is more abundant than the other (it is well known that PCR techniques normalise the quantity of transcripts when more than 35 rounds of amplification are used). We therefore think it probable that our approach can be applied to most samples from *PKD1* patients.

We found six mutations that are likely to be the cause of the disorder: a 4 bp insertion, three splice mutations, a 3 bp deletion, and one missense point mutation. Only one of the splice mutations has been previously identified. Moreover, using our approach we also detected a mutation in a sporadic case. Unfortunately, we have no access to the DNA of the parents, so we cannot know if this sporadic mutation is de novo or if the case is sporadic because of the small size of the family (a situation becoming more frequent in Europe with the decrease in family size). One of the splicing mutations (IVS45-14T>C) is interesting as it did not affect a consensus splice site, but affected the sequence of the intron. The only explanation for this (we have never found this splice variant in the either normal population or other patients) is that this mutation affects the secondary structure of the mRNA, necessary for the normal formation of the lariat in the first steps of splicing. These mutations did not correspond to the sequence of *PKD* homologues, ruling out the possibility of some non-specificity of our test.

Therefore, six mutations were detected in the nine patients. In spite of the small size of the tested population, this rate of success (67%) is higher than other published screening methods. This success is partially the result of the large number of spliced mutations detected in the assay (3), which can accurately be detected only by RT-PCR. However, this should be confirmed in a larger population. The failure to find mutations in 30% of patients may be because: (1) some of the patients were not *PKD1* patients; (2) SSCP screening does not have a 100% success rate and often gives false negative results; or (3) there was an error in the sequence of abnormal SSCP fragments. In order to increase our rate of success, we plan to use more sensitive and reliable approaches, such as mutation screening using the DHPLC technique.

In addition, we have detected numerous variations that appear to be polymorphisms, as they are present in both patients and unaffected subjects. These represent a high rate of nucleotide substitution. The most frequent base substitution in humans is C to T, which is also the case in the present study.

One explanation for this high rate of base substitution could be, as has already been proposed,[23 24] that gene conversion occurs between *PKD1* and related sequences, introducing mutations into *PKD1*. However, more than 60% of the amino acid or nucleotide changes detected in *PKD1* are not present in *PKD1* related transcribed sequences. Moreover, we may note that no putative mutations observed in our patients match the PKD related sequences. The gene conversion hypothesis cannot, therefore, explain the majority of sequence variants in the *PKD1* transcript. However, this observation supports our data, indicating that our technique specifically amplifies *PKD1* and not the related sequences. These findings also confirm the frequency of polymorphisms of *PKD1* previously described.[13 15] This surprisingly high rate of mutations/polymorphisms has so far not been elucidated. However, a high rate of mutations is consistent with the two hit strategy of cyst formation, as this theory implies a high rate of somatic mutations in order to have the chance to form a cyst.

In conclusion, we have developed a quick and reliable RT-PCR approach that specifically amplifies the whole *PKD1* transcript, allowing the screening of all types of mutations over the entire *PKD1* gene.

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REFERENCES

1 **Gabow PA**. Autosomal dominant polycystic kidney disease. *N Engl J Med* 1993;**329**:332-42.

2 **European** *PKD1* Consortium. The polycystic kidney disease 1 gene encodes a 14 kb transcript and lies within a duplicated region on chromosome 16. *Cell* 1994;**77**:881-94.
3 **International** *PKD1* Consortium. Polycystic kidney disease: the complete structure of the *PKD1* gene and its protein. *Cell* 1995;**81**:289-98.
4 **Burn TC**, Connors TD, Dackowski WR, Petry LR, Van Raay TJ, Millholland JM, Venet M, Miller G, Hakim RM, Landes GM, Klinger KW, Qian F, Onuchic LF, Watnick T, Germino GG, Dogget NA. Analysis of the genomic sequence for the autosomal dominant polycystic kidney disease (*PKD1*) gene predicts the presence of a leucine-rich repeat. The American *PKD1* Consortium. *Hum Mol Genet* 1995;**4**:575-82.
5 **Hughes J**, Ward CJ, Peral B, Aspinwall R, Clark K, San Millan JL, Gamble V, Harris PC. The polycystic kidney disease 1 (*PKD1*) gene encodes a novel protein with multiple cell recognition domains. *Nat Genet* 1995;**10**:151-60.
6 **Daalgard OZ**. Bilateral polycystic kidney disease of the kidneys a follow up of 284 patients and their families. *Acta Med Scand* 1957;**158**:1-255.
7 **Loftus BJ**, Kim UJ, Sneddon VP, Kalush F, Brandon R, Fuhrmann J, Mason T, Crosby ML, Barnstead M, Cronin L, Deslattes Mays A, Cao Y, Xu RX, Kang HL, Mitchell S, Eichler EE, Harris PC, Venter JC, Adams MD. Genome duplications and other features in 12 Mb of DNA sequence from human chromosome 16p and 16q. *Genomics* 1999;**60**:295-308.
8 **Bogdanova N**, Markoff A, Gerke V, McCluskey M, Horst J, Dworniczak B. Homologues of the first gene for autosomal dominant polycystic kidney diseases are pseudogenes. *Genomics* 2001;**74**:331-41.
9 **Peral B**, Gamble V, Strong C, Ong AC, Sloane-Stanley J, Zerres K, Winearls CG, Harris PC. Identification of mutations in the duplicated region of the polycystic kidney disease 1 gene (PKD1) by a novel approach. *Am J Hum Genet* 1997;**60**:1399-410.
10 **Peral B**, San Millan JL, Ong AC, Gamble V, Ward CJ, Strong C, Harris PC. Screening the 3′ region of the polycystic kidney disease 1 (PKD1) gene reveals six novel mutations. *Am J Hum Genet* 1996;**58**:86-96.
11 **Roelfsema JH**, Spruit L, Saris JJ, Chang P, Pirson Y, van Ommen GJ, Peters DJ, Breuning MH. Mutation detection in the repeated part of the PKD1 gene. *Am J Hum Genet* 1997;**61**:1044-52.
12 **Thongnoppakhun A**, Rungroj N, Wilairat P, Vareesangthip K, Sirinavin C, Yenchitsomanus PT. A novel splice-acceptor site mutation (IVS13-2A>T) of polycystic kidney disease 1 (*PKD1*) gene resulting in an RNA processing defect with a 74-nucleotide deletion in exon 14 of the mRNA transcript. *Hum Mutat* 2000;**15**:115.
13 **Rossetti S**, Strmecki L, Gamble V, Burton S, Sneddon V, Peral B, Roy S, Bakkaloglu A, Komel R, Winearls CG, Harris PC. Mutation analysis of the entire *PKD1* gene: genetic and diagnostic implications. *Am J Hum Genet* 2001;**68**:46-63.
14 **Watnick TJ**, Gandolph MA, Weber H, Neumann HP, Germino GG. Gene conversion is a likely cause of mutation in *PKD1*. *Hum Mol Genet* 1998;**7**:1239-43.
15 **Thomas R**, McConnell R, Whittacker J, Kirkpatrick P, Bradley J, Sandford R. Identification of mutations in the repeated part of the autosomal dominant polycystic kidney disease type 1 gene, *PKD1*, by long-range PCR. *Am J Hum Genet* 1999;**65**:39-49.
16 **Watnick TJ** , Phakdeekitcharoen B, Johnson A, Gandolph M Wang M, Briefel G, Klinger KW, Kimberling W, Gabow P, Germino GG. Mutation detections of *PKD1* identifies a novel mutation common to three families with aneurysms and or very early onset disease. *Am J Hum Genet* 1999;**65**:1561-71.
17 **Phakdeekitcharoen B**, Watnick TJ, Ahn C, Whang DY, Burkhart B, Germino GG. Thirteen novel mutations of the replicated region of *PKD1* in an Asian population. *Kidney Int* 2000;**58**:1400-12.
18 **Perrichot R**, Mercier B, Quere I, Carre A, Simon P, Whebe B, Cledes J, Ferec C. Novel mutations in the duplicated region of *PKD1* gene. *Eur J Hum Genet* 2000;**8**:353-9.
19 **Aguiari G**, Savelli S, Garbo M, Bozza A, Augello G, Penolazzi L, De Paoli Vitali E, La Torre C, Cappelli G, Piva R, del Senno L. Novel splicing and missense mutations in autosomal dominant polycystic kinase disease 1 (*PKD1*) gene: expression of mutated genes. *Hum Mutat* 2000;**16**:444-5.
20 **Perrichot RA**, Mercier B, Simon PM, Whebe B, Cledes J, Ferec C. DGGE screening of *PKD1* gene reveals novel mutations in a large cohort of 146 unrelated patients. *Hum Genet* 1999;**105**:231-9.
21 **Badenas C**, Torra R, San Millan JL, Lucero L, Mila M, Estivill X, Darnell A. Mutational analysis within the 3′ region of the *PKD1* gene. *Kidney Int* 1999;**55**:1225-33.
22 **Bateman A**, Sandford R. The PLAT domain: a new piece in the *PKD1* puzzle. *Curr Biol* 1999;**26**:R588-90.
23 **Daniells C**, Maheshwar M, Lazarou L, Davies F, Coles G, Ravine D. Novel and recurrent mutations in the *PKD1* (polycystic kidney disease) gene. *Hum Genet* 1998;**102**:216-20.
24 **Afzal AR**, Hand M, Ternes-Pereira E, Saggar-Malik A, Taylor R, Jeffery S. Novel mutations in the 3′ region of the polycystic kidney disease 1 (*PKD1*) gene. *Hum Genet* 1999;**105**:648-53.