Co-inheritance of a PKD1 mutation and homozygous PKD2 variant: a potential modifier in autosomal dominant polycystic kidney disease

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ABSTRACT

Background Autosomal dominant polycystic kidney disease (ADPKD), which is caused by mutations in polycystins 1 (PC1) and 2 (PC2), is one of the most commonly inherited renal diseases, affecting ~1 : 1000 Caucasians.

Materials and methods We screened Greek ADPKD patients with the denaturing gradient gel electrophoresis (DGGE) assay and direct sequencing.

Results We identified a patient homozygous for a nucleotide change c.1445T > G, resulting in a novel homozygous substitution of the non-polar hydrophobic phenylalanine to the polar hydrophilic cysteine in exon 6 at codon 482 (p.F482C) of the PKD2 gene and a de-novo PKD1 splice-site variant IVS21–2delAG. We did not find this PKD2 variant in a screen of 280 chromosomes of healthy subjects, supporting its pathogenicity. The proband's parents did not have the PKD1 mutation. Real-time PCR of the PKD2 transcript from a skin biopsy revealed 20-fold higher expression in the patient than in a healthy subject and was higher in the patient's peripheral blood mononuclear cells (PBMCs) than in those of her heterozygote daughter and a healthy subject. The greater gene expression was also supported by Western blotting. Inner medullar collecting duct (IMCD) cells transfected with the mutant PKD2 mouse gene presented a perinuclear and diffuse cytoplasmic localization compared with the wild type ER localization. Patch-clamping of PBMCs from the p.F482C homozygous and heterozygous subjects revealed lower polycystin-2 channel function than in controls.

Conclusions We report for the first time a patient with ADPKD who is heterozygous for a de novo PKD1 variant and homozygous for a novel PKD2 mutation.

Keywords Channel activity, mutation, PKD, polycystins.

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Introduction

Autosomal dominant polycystic kidney disease (ADPKD) is one of the most common inherited disorders, with an incidence of 1/1000 [1] and accounts for 8–10% of all cases of end-stage renal failure (ESRF) in western Europe [2]. The disease is characterized primarily by the formation and enlargement of multiple cysts in both kidneys, which can lead to ESRF in adults. Hypertension, nephrolithiasis, macroscopic haematuria, cardiac valve abnormalities and cerebrovascular aneurysms are also some of the clinical symptoms in families with ADPKD [3]. In the majority (> 85%) ADPKD is associated with germline mutations in PKD1

(MIM#601313) on chromosome 16p13.3, while for most of the remaining cases with PKD2 (MIM#173910) on chromosome 4q21-q23 [4]. Still, for some patients no chromosomal location has been described [5,6]. A pair of genes homologous to PKD1 has been recently identified [7].

The PKD1 gene encodes a large (460 kDa), integral membrane multi-domain protein, polycystin-1, with an extensive extracellular region and with a variety of characterized domains of homology with other proteins [8–10]. The PKD2 gene, located on chromosome 4, spans approximately 68 kbp and

encodes a 5·4-kb transcript. The protein resulting, polycystin-2, a non-selective cation channel with permeability to calcium [11,12] is an integral membrane of 968 amino acids and of 110 kDa, with six putative transmembrane spanning domains and intracellular NH2- and COOH-termini [4]. Different groups using independent approaches have identified direct interactions between the two proteins, mediated by the coiled-coil domain of polycystin-1 and the cytoplasmic COOH-terminus of polycystin-2 [13,14]. Polycystin-1 and polycystin-2, act non-redundantly as a receptor-channel complex [15] in a signalling pathway that is essential to the formation and maintenance of the highly differentiated state of lumen-forming epithelia in the kidney, liver and pancreas. Alterations in polycystin signalling that result in polycystic kidney disease affect proliferation, apoptosis, polarization and transcellular transport properties of renal epithelial cells [16].

Presymptomatic diagnosis of ADPKD is possible by various imaging methods (abdominal ultrasound, computer tomography and magnetic resonance). However, there is a role for genetic diagnosis in this disorder, especially in patients with equivocal imaging results or with a negative family history, or in young relatives and potential kidney donors.

Mutation analysis would be a direct method of genetic diagnosis and would allow study of genotype/phenotype correlations. However, mutation screening is difficult for the PKD1 gene since it encodes 46 exons and is embedded in a complex duplicated genomic area [1,8]. On the contrary, PKD2 comprises 15 exons extending to a region of about 68 kb. Various methods [17–19] have been applied so far for PKD2 mutation screening. We designed primers, PCR and electrophoresis conditions to use the denaturing gradient gel electrophoresis (DGGE) assay to screen for mutations in the PKD2 gene in Greek patients with ADPKD.

Materials and methods

Study subjects

An ADPKD patient and two generations of her family members were included in this study. The diagnosis of the disease was confirmed by reviewing her clinical records and was established by a nephrologist and supported by widely used criteria [20] i.e. the presence of at least three bilateral cysts. For renal imaging modalities, the patient and her family members were subjected to ultrasonography. Abdominal NMR was also performed to evaluate the presence of intracranian aneurysms. Mitral valve prolapse and mitral regurgitation were excluded, as previously described [21]. The family pedigrees and clinical data, including phenotype and age of ESRF were collected. All participants gave their written consent after a full explanation of the study. The study was approved by the Harokopio University Bioethics Committee.

Blood samples were obtained and genomic DNA was extracted from peripheral blood mononuclear cells (PBMCs) following a standard procedure. DNA was isolated from a bone marrow specimen from the dead father with the Qiagen tissue extraction kit (Qiagen, Valencia, CA, USA) following the manufacturer's guidelines.

Mutation analysis

Genomic DNA was amplified using standard methods. For amplification of bone marrow DNA, and after we tried different Taq polymerases unsuccessfully, we obtained a visible PCR product with the HotStarTaq DNA polymerase (Qiagen, Valencia, CA, USA). The primers used for DGGE were designed by the WinMelt program (BIO-RAD Laboratories, CA, USA) and are listed in Table 1, together with the size of the PCR product, annealing temperature, gradient range and DGGE running time. The PCR products were electrophoresed on a BIO-RAD Decode system. Primer sequences and PCR conditions for PKD1 gene were described previously [22].

Sequence analysis

The PCR programs were purified with the QIAquick PCR spin purification kit (Qiagen) and were automatically sequenced by the Harvard Medical School sequencing service (http://dnaseq.med.harvard.edu). Alignment analysis was performed with ClustalW (http://clustalw.genome.ad.jp/) [accessed 31 August 2004].

Real-time PCR analysis

Total RNA was harvested from PBMCs and skin tissue with the Trizol (Invitrogen, Carlsbad, CA, USA) and the RNeasy Lipid Tissue Midi (Qiagen) kits, respectively, according to the suppliers' protocols. RNA quality and concentration were assayed by Agarose gel electrophoresis and UV spectrophotometry (A260/A280). Five micrograms of total RNA was reverse transcribed with Superscript II reverse transcriptase (Invitrogen) in the presence of RNAseOUT™, a ribonuclease inhibitor (Invitrogen). Samples were prepared with the Quantitect SYBR green kit (Qiagen) according to the manufacturer's protocol, and semiquantitative real-time PCR for PC2 was performed on a Lightcycler 1 (Roche, Indianapolis, IN, USA) with GADPH as an internal control. The following primer sets for mouse pc2 (GenBank NM_008861) and GADPH (GenBank M32599) were designed with Vector NTI software (Informax) using identical criteria: pc2 5'-TGTGTGGTCAGGTTATTGGCG, 3'-GCCAGGAAGAAATCAAAGGC. GADPH 5'-GATGCCCCC-ATGTTTGTGAT, 3'-TTGTCATGGATGACCTTGGC. Real-time PCR was performed using the following temperature profile: Denature: 95 °C, 15 min; Cycle: 94 °C, 15 s; 53 °C, 8 s; 72 °C, 8 s.

Melting curve analysis was performed after each run, and data were analyzed with Roche light-cycler data analysis software (v3·5.28). ΔcT values for GADPH and Polycystin

Table 1 Primers and PCR-DGGE parameters of PKD2 gene

#Exon	Forward (5'– 3')	Reverse (5'– 3')	Annealing Temp ℃	Size (bp)	Gradient (%)	Time (h)
Ex1·1	CCGAGCGCGGCGCCGCGCAC	*AAGCCGGGGTTATCGCGGCT	67	367	30–70	18
Ex1·2	†CGAGGCCGAGGAGGAGGAGGA	GGCGCCGTGCATCTGCCGCTGCGGGT	67·7	400	30–70	18
EX2	*TTCTTAAATAAAATGATATC	ATCGTTTCAAAACTTTCCCA	50·2	248	20–40	16
EX3	GTGTGCCGGTTCCCTTGGGG	‡TGTGATAGAGAGGTACTTTC	55	260	20–60	18
EX4	‡CAGGGCAAAGCAGCGGCATG	CACCGAGTGCCAATGAGTCA	56·2	371	20–60	18
EX5	§GGCCTCAAGTGTTCCACTGAT	AGGAGGGATGCATGTCCTCA	58·5	349	20–60	18
EX6	†TTGTTATTGTTTTAATTGTT	¶TAGAATAGGAAATTTGGTG	56·2	347	20–50	18
EX7	†CATATTTCTAAAACACTGTA	¶TgTAgAACATgAACATgAAATTTATA	55	291	20–50	18
Ex8	*ACACCATTTGGTTTATCCATTC	CTTAATTTCATATATACTTACATAC	60	294	20–60	18
Ex9	*TGGTGTTTGTTTATTTTTATAGCTTC	CCACTATAAATATAAATGTACATACC	66	209	30–70	16
Ex10	TTGATTGATAATTCCAAATTATGTTTC	*TTTGAGAATTAAGGTTTTTCCTACC	66	228	30–70	16
Ex11	†CTAAACCAAgTCTTTTATTT	¶gAgCCCTTgggCTAgAAAATA	52	295	20–40	18
Ex12	GGATGAATGTTATCTGTATCCTC	*TGTGGTGTTGTAGGTACCAAATC	65	246	30–70	18
Ex13	*TTCTGTGGGGTCTCAGTGTTC	TCACGCAAATTCTGCCAATTCC	64	291	20–60	18
Ex14	*GTACTGTGTTTTCCTTGCAGC	TGTCAGCAACATATATTATTAATAAC	65	275	30–70	18
Ex15	CCTTTTTAGGATGAAAGGCTGG	*CTTCCATGCCTTCTGTAGATTG	64	249	20–60	18

*: 40 bp GC-clamp CgCCCgCCgCgCCCCgCgCCCgTCCCgCCgCCCCCgCCCg.
†: 44 bp GC-clamp ggCggCgCCCgCCgCgCCCCgCgCCCgTCCCgCgCCCCCgCCCg.
‡: 36 bp GC-clamp CgCCCgCCgCgCCCCgCgCCCgTCCCgCCgCCCCCg.
§: 35 bp GC-clamp CgCCCgCCgCgCCCCgCgCCCgTCCCgCCgCCCCC.
¶: 10 bp GC-clamp CCgCgggC.

2 were determined by second-derivative analysis of the amplification curves. ΔcT for GADPH was used to normalize PC2 data (subtracted from ΔcT for PC2), and final results were expressed as percentages of the control value.

DNA constructs

To obtain Pkd2-pcDNA4 construct, full-length mouse Pkd2 cDNA was previously cloned into pcDNA4/TO/Myc-His mammalian expression vector (Invitrogen) [12]. QuikChange II XL Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA, USA) was used to point mutate wild-type Pkd2 cDNA in this construct at t1435g. The resulting mutant construct Pkd2-F480C-pcDNA4 mimicked the homozygous F482C PKD2 mutation found in this patient.

Cell culture and transient and stable transfections

IMCD (ATCC catalogue no.CRL-2123) cells were cultured in Dulbecco's modified Eagle's medium/F12 medium supplemented with 10% (v/v) fetal bovine serum (Invitrogen, CA, USA). HEK293-S-PC1 (stably expressing Polycystin 1) cell lines were cultured in DMEM medium with 10% (v/v) fetal bovine serum, Zeocin (200 mg mL^{-1}) and blasticidin (5 mg mL^{-1}) (Invitrogen). For induction of PC1 expression,

1 mg mL^{-1} of tetracycline was added to the medium. Transient transfections were carried out on cells cultured to 30% to 50% confluence. DNA constructs were transfected with Fugene 6 transfection reagent (Roche) following the manufacturer's protocol. Forty-eight hours after the transfection, cells were harvested for further analysis.

Antibodies

Affinity-purified polyclonal antibodies MR3 and 96525 were raised in rabbits to 2938–2956 amino acids of human PC1 and 44–62 amino acids of mouse PC2, respectively [12,23,24]. Purified antibody was used at a 1 : 500 dilution for immunofluorescence, 1 : 50 for immunoprecipitation, and 1 : 1000 for Western blotting. Mouse monoclonal antibody to the Myc tag (Invitrogen) was used at 1 : 500 for immunoprecipitation and immunofluorescence. Mouse monoclonal antibody to the His tag (Santa Cruz Biotechnology, Santa Cruz, CA, USA) at a 1 : 5000 dilution was used for Western blotting. Goat antirabbit immunoglobulin G (IgG)-fluorescein isothiocyanate (FITC) and goat antimouse IgG-PE (Vector) were used as secondary antibodies for immunofluorescence at a dilution of 1 : 500. For Western blotting, goat antirabbit IgG-horseradish peroxidase and goat antimouse

IgG-horseradish peroxidase, 1 : 10 000 dilution (Amersham Pharmacia Biotech, NJ, USA), were used as secondary antibodies.

Co-immunoprecipitation and Western blotting

HEK293-S-PC1 cells were grown to semi-confluence. Cells were transfected with either Pkd2-pcDNA4 or Pkd2-F480C-pcDNA4 construct. After 48 h, cells were harvested and lysed. Anti-PC1 antibody 96521 was used to immunoprecipitate the PC1 proteins in cell lysate, and antihis antibody was used to specifically blot transiently expressed PC2 proteins coimmunoprecipitated by PC1. Co-immunoprecipitation and Western blotting were applied as described previously [12]. The relative levels of PC2 were measured by densitometry of PC2 bands by an imaging densitometer (BioRad Laboratories, Hercules, CA, USA) and normalized to β-actin.

Immunofluorescence

Immunofluorescence on IMCD cells was performed as previously described [12]. Prolonged mounting medium (Molecular Probes, Invitrogene, CA, USA) was used to protect immunofluorescence signals from fading. A Zeiss Axioskop2 Plus fluorescence microscope (Carl Zeiss, NY, USA) was used to observe the signal and the Spot camera system (Diagnostic Instruments) was used for photography. An Ultra-View spinning-disc confocal scanner (Perkin-Elmer, UK), Nikon TE2000-U microscope (Nikon, NY, USA), and Hamamatsu ORCA-ER camera (Hamamatsu, Japan) were used to capture images when noted.

Electrophysiological techniques

PBMC isolated from the patient, different family members and healthy donors, were centrifuged for 10 min at 5000 r.p.m. Cells were electrically characterized immediately after arrival or frozen for later use. Prior to electrical characterization, cells were placed onto glass coverslips coated with poly L-lysine using standard procedures (1 : 10 dilution, Sigma) and dried overnight. Freshly isolated cells were also placed on naked coverslips, which normally moved and were more difficult, although not impossible, to patch. Aliquots of either fresh or thawed cells were allowed to settle onto the poly L-lysine-coated coverslip for several minutes prior to washing directly into the patch-clamping chamber. Cells were identified under phase contrast (×60) with an Olympus IMT-2 inverted microscope.

Patch-clamping technique

To determine potentially relevant functional differences in the PC2 channel activity of all samples, PBMCs attached and excised inside-out patches were obtained from control donors and from both heterozygotes and homozygotes for the F482C mutation. Patch-clamping techniques were conducted as previously reported [11,23,25]. Briefly, single cells were first patched under cell-attachment conditions and immediately attached into the

excised inside-out patch-clamping configuration. The patch pipette was filled with a solution containing, in mM: 140 K^+ aspartate Cl, 5·0 KCl, 1·0 $MgCl_2$, 2·5 $CaCl_2$, and 10 HEPES, adjusted to pH 7·4 with N-methylglucamine. The bathing solution containing, in mM: 145 KCl, 1·0 $MgCl_2$, 2·5 $CaCl_2$, and 10 HEPES, adjusted to pH 7·4 with N-methylglucamine. EGTA (1 mM) was added from a 100 mM stock solution. Currents and command voltages were obtained with a Dagan 3900 patch-clamp amplifier (Dagan) under voltage clamp configuration. All electrical signals were filtered at 5 kHz with the internal four-pole Bessel filter (Dagan). Single channel currents were further filtered digitally at 200 Hz for graphic purposes only. Mean versus variance analysis of single channel subconductance states was conducted as previously reported [12]. Chloride channel contamination was a constant. Thus, K^+ channel activity was confirmed as channel activity in which no current was observed at 0 mV and also at the reversal potential for K^+. PC2 channel activity was inhibited with the rabbit anti-PC2 antibody (Zymed Laboratories, #52–5217). The antibody was added to the cytoplasmic side of excised inside-out patches from a 0·2-mg mL^{-1} stock solution to 1 : 6000–30 000 dilutions, at a final concentration of 3–7 ng mL^{-1}. Data were analyzed with either PClamp 6·0.3 (Axon Instruments) or Axograph 4·0 (Axon Instruments).

Results

Clinical characteristics

The pedigree of the family is plotted in Fig. 1. The proband was found to be homozygous for a missense variant in the PKD2 gene and heterozygous for a splice-site variant in PKD1. She was born in 1953; she initially presented at the hospital with hypertension, fatigue, loin pain and severe uraemia. She was diagnosed with PKD when she was 39 years old; she entered at the ESRF at the age of 41 years. Ultrasound analysis found multiple cysts (> 3) in both kidneys. No other extrarenal cysts were observed by NMR (data not shown). Patient was also experiencing repeated severe
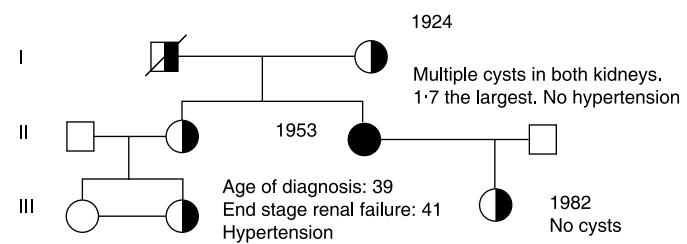

Figure 1 Pedigree analysis of the polycystic kidney disease (PKD) family. The patient II3 was found to be homozygous for a missense variant in the PKD2 gene and heterozygous for a splice-site variant in PKD1. See text for patient and family history.
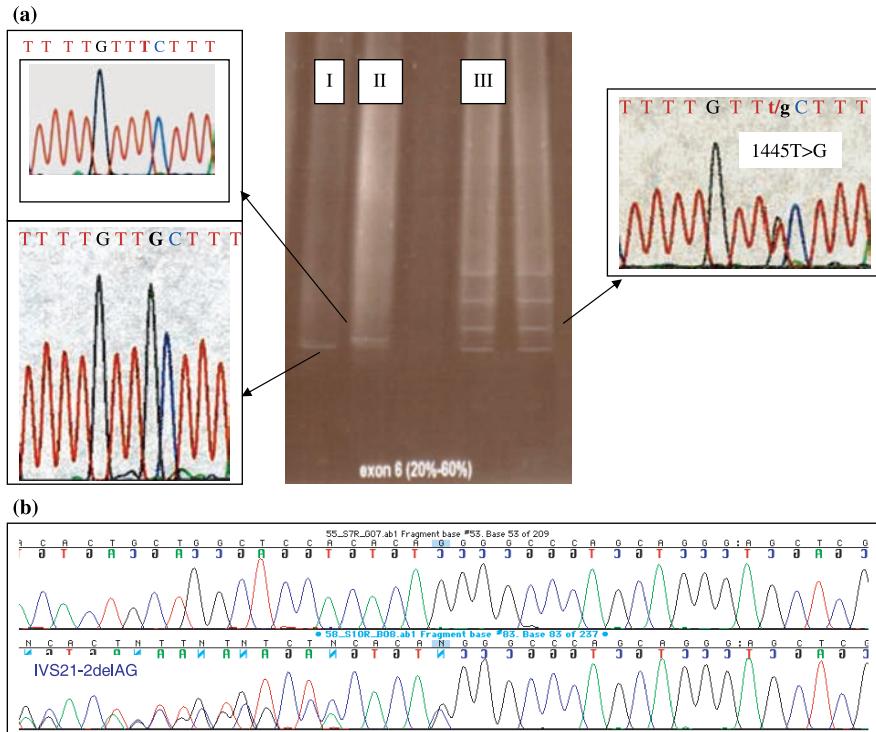
(a)

(b)

Figure 2 Denaturating gradient gel electrophoresis (DGGE) and sequencing results in *PKD1* and *PKD2* genes. (a) (I) F482C in the homozygous form (II); wild-type sequence (III and IV); and F482C in the heterozygous form. (b) The proband was found to be heterozygous for the splice-site variant IVS21–2delAG in the *PKD1* gene.

cyst infections, while her serum creatinine level was in the range of 12–13 mg dL^{-1}. At the age of 51 years she was successfully transplanted with a kidney donated from a cadaver donor. All of her relatives underwent ultrasound analysis. Her healthy sister has no symptoms of the disease. Her mother, who was born in 1924, is healthy with no symptoms of the disease. Multiple cysts, less than 10, were found on both her kidneys (the largest was 1·7 cm), with no hypertension. All the biochemical parameters of kidney function were at normal levels (data not shown). The proband's father died from colon cancer in 1984. He had no symptoms of ADPKD. According to the proband's testimony, her parents were not relatives and came from different cities in the Western part of Greece. Her daughter was born in 1982 and has no symptoms of the disease.

Mutation analysis

We are reporting primer design, PCR conditions, and electrophoretic parameters (Table 1) for DGGE analysis of the PKD2 gene for the first time. After screening all exons of the *PKD2* gene by DGGE in 30 unrelated patients with PKD, we identified a novel missense variant in exon 6 (Fig. 2). This mutation was a homozygous substitution of a T to G at 1445 resulting in a substitution of phenylalanine (Phe) to cysteine (Cys) *et al.* amino acid position 482. We then sequenced all exons of the PKD2 gene and we did not find any other variant. In addition, the proband was found to be heterozygous for the splice-site variant in IVS21–2delAG. Screening other members

of the family, including her father's DNA isolated from a bone marrow specimen, confirmed the co-segregation of the PKD2 variant. On the other hand, PKD1 was absent in her parents, thus annotating the PKD1 variant as a de-novo mutation. To determine if the PKD2 variant is present in healthy subjects, we sequenced 280 chromosomes and we found that all of them had the wild-type sequence, indicating that this variant could be considered a pathogenic polymorphism.

Expression and localization of PC2 in human skin

To verify that the mutation was present in different tissues, we sequenced the skin-tissue DNA of both a healthy subject and the patient. The patient's was found to be homozygous for the Cys (p.C482C), whereas the healthy subject was homozygous for the Phe (p.F482F) (data not shown). To evaluate the relative amount of PKD2 RNA precisely, we performed real-time RT-PCR for the skin-tissue samples. The comparative ΔC_T method with GADPH as an endogenous reference for normalization was used (the higher the ΔC_T value, the lower the initial cDNA content). ΔC_T mean values were lower in patient skin tissue than in normal skin (7·44 ± 1·8 vs. 11·8 ± 3·7), indicating a constantly higher content in PKD2 skin RNA from the F482C homozygote. Real-time analysis (Fig. 3a), expressed as relative quantification, showed that levels of PKD2 expression were approximately 20 times higher in the patient's skin than in the healthy subject's skin. When a similar experiment was performed in PBMC, PKD2 ΔCT mean values were found to be lower in the patient than in her
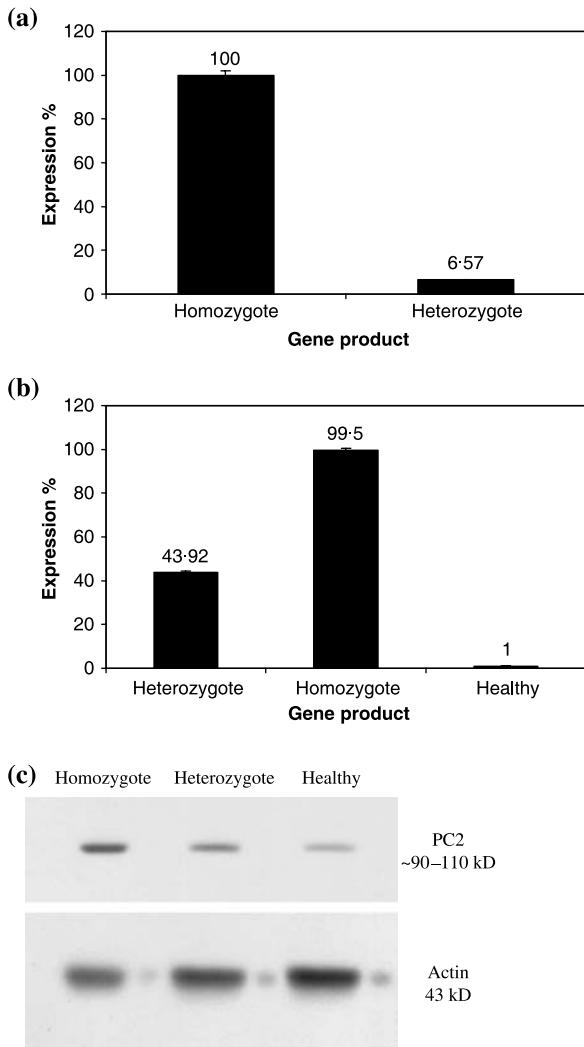
(a)

(b)

(c)

Figure 3 Total mRNA was harvested from peripheral blood mononuclear cells (PBMCs) and skin tissue. Total RNA was reverse transcribed, and semiquantitative real-time PCR for Polycystin 2 (PC2) was performed with GADPH as an internal control. ΔcT for GAPDH was used to normalize pc2 data (subtracted from ΔcT for pc2), and final results were expressed as a percentage of control. Each PCR repeated three times from two separate experiments. (a) mRNA expression of PKD-2 gene in human skin. The mRNA content in PKD2 skin RNA from the F482C homozygous was consistently higher than in control skin. (b) m-RNA expression of PKD-2 gene in PBMCs. Real-time analysis, expressed in terms of relative quantification, showed higher expression in the F482C homozygote than in the F482C heterozygote and a healthy donor. (c) Western blot analysis of total cell extracts with anti-PC2 antibodies showing that PC2 is detectable as a band of ~110 kDa in kidney cells. By densitometry of PC2 bands and normalized to β-actin in PBMC, the relative expression of PC2 was 4 and 2·5 times lower in a healthy donor and in a heterozygote, respectively, than in the homozygote.
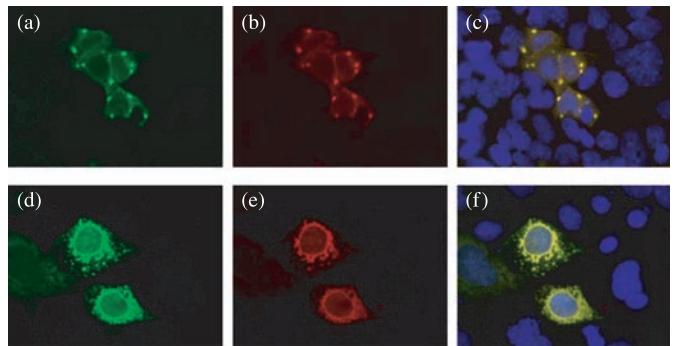

Figure 4 Fluorescence microscopy of transient transfected IMCD cells with mouse PKD2 gene (×100). (a–c) Wild-type construct. (a) Cells stained with anti-PC2 FITC. (b) Cells stained with anti-Myc PE. (c) Merge. D-F) Mutant construct with the F480C mutation. (d) Cells stained with anti-PC2 FITC. (e) Cells stained with anti-Myc PE. (f) Merge. Note the diffuse cytoplasmic and perinuclear staining of IMCD cells transfected with the mutant construct.

heterozygote daughter, while both of them had lower ΔCT mean values than a healthy subject (4·8 ± 0·15 vs. 6 ± 0·01 vs. 11·44 ± 0·7). Real-time analysis (Fig. 3b), expressed as relative quantification, showed increased levels of expression in the F482C homozygote (approximately 2·3 times higher) than in her heterozygote daughter and 100 times higher than in a healthy subject.

Western blot analysis of total cell extracts with anti-PC2 antibodies showed that PC2 was detectable as a band of ~110 kDa reported also for kidney cells (Fig. 3c). By densitometry of PC2 bands and normalized to β-actin in PBMC, the relative expression of PC2 was 2·5 times lower in that heterozygote and 4 times lower in a healthy subject than in the homozygote.

PC2 and PC1 were both expressed on the skin sections of both the healthy donor and the patient, as revealed by immunofluorescence (data not shown). A higher intensity and perinuclear staining were more pronounced in the section from the patient. These findings were confirmed by confocal microscopy. To further evaluate a possible dissimilar localization of the mutant protein than of the normal protein, we generated constructs bearing the normal or the mutant PC2 gene and subsequently transfected them into IMCD cells. Immunofluorescence analysis showed that the majority of mutant PC2 cells had diffuse cytoplasmic and perinuclear staining (Fig. 4), whereas most of the wild-type cells presented a well-localized staining in the ER.

Co-immunoprecipitation assay

To determine if the presence of the mutation affected binding of the PC1, we performed the coimmunoprecipitation assay. The wild-type and the mutant PC2 proteins coimmunoprecipitated equally (Fig. 5).
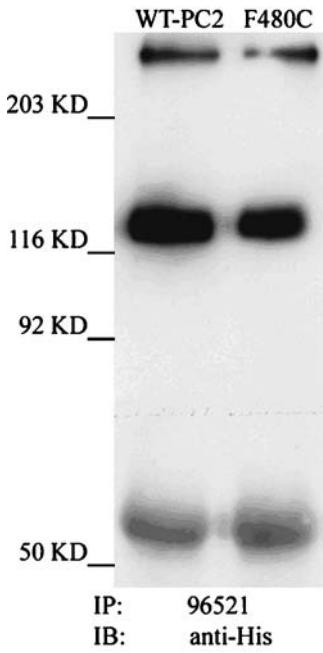
Figure 5 Co-immunoprecipitation and Western blotting assay. HEK293-S-PC1 cells were grown to semiconfluence. Cells were transfected with either Pkd2-pcDNA4 or Pkd2-F480C-pcDNA4 construct. After 48 h, cells were harvested, and lysed. Anti-PC1 antibody 96 521 was used to immunoprecipitate the PC1 proteins in cell lysate, and antihis antibody was used to specifically blot transient expressed PC2 proteins coimmunoprecipitated by PC1. The wild-type and the mutant PC-2 proteins coimmunoprecipitated equally.

PC2 channel activity in PBMC

To determine differences in PC2 cation channel activity from the different donors, we obtained electrical information from either fresh or freshly thawed frozen cells. Three criteria were used to assess the presence of a functional PC2 channel. First, strictly cation-selective channel activity was identified in a KCl/K^+-aspartate gradient. Second, the K^+-permeable channels had a maximal conductance of ~150 pS and displayed multiple subconductance states [23]. Third, channel activity was selectively inhibited with an anti-PC2 antibody (Zymed) as recently reported [25]. Cells were patched in a blind fashion. PC2 channel activity from heterozygous donors ($n = 15$) was almost identical to that of the homozygous patient ($n = 7$) and to excised inside-out patches of control donors ($n = 9$). All cells that showed PC2 channel activity as defined by the first two criteria (i.e. high conductance in asymmetrical K^+ and the presence of subconductance states) were tested for inhibition by an anti-PC2 antibody (Zymed), which inhibits PC2 but not Cl^- permeable channel function [25]. Addition of the antibody

completely blunted PC2 channel function in all groups tested (Fig. 6). Mutant PC2 in both the heterozygous and homozygous donors was active and reached maximal conductance, but only infrequently (Fig. 7). Thus, the F482C mutation affects the kinetic properties of PC2 but not its maximal conductance. The most frequent level in the mutant channel is the lowest possible conductance, ~30–40 pS, most likely due to dysfunctional assembly (Fig. 7).

Alignment analysis

To study if the F482C variant concerns a conservative residue, we performed alignment analysis (Fig. 8). To compare the results with a known pathogenic mutation, we present in the same figure the alignment analysis of the D511V mutation. Both variants were found to be conservative among vertebrates so they might be important for protein structure or function. On the other hand D511V was found to be more conservative among species, thus underlying its pathogenicity.

Discussion

We describe for the first time a patient with PKD who was homozygous for a missense variant in the PKD2 gene and also heterozygous for a de-novo PKD1 splice-site variant. Although the main pathogenic variant seemed to be the PKD1 splice-site deletion, her phenotype was more severe than that for PKD1. This could be attributed to her homozygous PC2 mutated protein.

Previously shown [26] were two individuals with *trans*-heterozygous mutations involving both genes and with renal disease more severe than that in individuals with either one mutation. Those patients developed ESRD at the ages of 48 years and of 52 years, respectively. PKD2 is associated with a less severe course of the disease (onset of the end-stage renal disease occurs at the age of 53 years for PKD1 compared to 69 years for PKD2) [27,28]. Because of the milder phenotype of ADPKD2 disease, many carriers of a PKD2 mutation remain free of obvious diagnostic symptomatology until death, permitting the assumption that ADPKD2 disease is more prevalent than generally thought. This could explain the absence of diagnosed disease in her 83 year-old mother.

PKD2 patients are at risk of vascular complications of ADPKD; intracranial aneurysms have been identified in PKD2 families. Systemic hypertension is also very common, occurring in > 75% of patients. Our patient has hypertension but no aneurysm has been diagnosed. The position but not the type of germline mutation to PKD1 has recently been shown to be an important factor that influences the likelihood to develop an aneurysm [29]. High rates of 5′ (NH_2-terminal) mutations were associated with vascular pedigrees. PKD1 splice variant produces a deletion of 13 amino acids (del2673–2686) [19]; thus, according to the ROC analysis, this mutation would have a reduced likelihood of
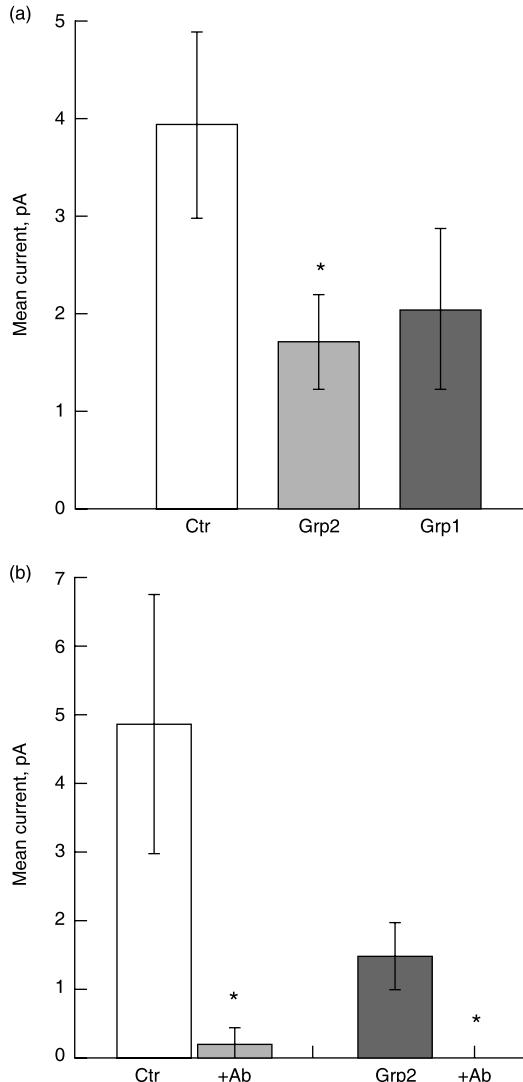
Figure 6 Averaged data and effect of anti-PC2 antibody on wild-type and mutant PC2 single channel currents. Top: Mean currents of excised inside-out patches for wild-type PC2, the homozygous donor (Group 1), and a heterozygous donor (Group 2). Data are the means ± SEM for 6, 2, and 13 cells, respectively. The value for the homozygous donor is almost identical to that of the heterozygous group but does not reach statistical significance. Bottom: Averaged mean currents for controls (PC2) and a heterozygous donor (Group 2), showing that both populations of channels are completely inhibited by addition of anti-PC2 antibody to the bathing solution under excised inside-out conditions. Maximal effect was obtained at 1–5 min. Data are the mean ± SEM of seven and four experiments, respectively. Asterisks indicate statistical significance to $P < 0.05$.

leading to the development of an intracranial aneurysm. Although 50% of the ADPKD patients bearing a mutation exhibit extrarenal abnormalities, our compound PKD1-PKD2 hetero-homozygous patient had normal extrarenal characteristics.

ADPKD is a focal disease that involves only a small fraction of the cells in the kidney, even though all cells carry one copy of the mutated gene [30]. Homozygous polycystin-1 and –2 knockout mice die *in utero* but exhibit gross polycystic renal disease, consistent with a loss-of-function mechanism for ADPKD [31]. Our patient survived, even despite her compound PKD1-PKD2 hetero-homozygosity. The germline PKD2 homozygous mutation in our patient was also confirmed in DNA isolated from her skin lesion (data not shown).

To exclude the possibility that the missense PKD2 variant on both of her alleles is a polymorphism, we screened 280 chromosomes from Greek healthy subjects originating from different areas of Greece and the mutated PKD2 allele was found in none of them. Reported molecular analyses on Greek PKD patients are limited [32,33]. All of the studies revealed mutations in the PKD1 gene [34]; another reported a de-novo variant located in the REJ module in the long extracellular domain that was similar to our PKD1 variant.

Several laboratories have reported ~200 different PKD1 mutations and > 60 PKD2 mutations [34]. Only 5% of PKD2 mutations are missense variants. Our missense variant is located in the second transmembrane domain (TM2) [35] being part of the calcium sensor. Another published [36] missense mutation is R322Q in the N-terminal region just before TM1. This position is conserved in all PKD2-related sequences, including *Caenorhabditis elegans*, suggesting that it is of critical functional or structural importance. That patient was 41 years old at the time of diagnosis, the same age as our patient. Another pathogenic missense mutation, D511V that is located in the third membrane-spanning domain presented a pattern of expression indistinguishable from the wild-type PC-2 [37]. Wild-type PC-2 was overexpressed twofold, finally resulting in loss of function in its channel properties. We measured both the PKD2 RNA and protein levels from PBMC. The transcription and the translation expression level were significantly higher in the patient than in her heterozygote daughter and in a healthy subject. That D511V mutation is more conservative than F482C among species (Fig. 8) although F482C variant might be important for protein structure or function, as it is conserved in all vertebrate polycystin-2 proteins. On the other hand, the preservation of the D511V residue through the evolution process could explain the severity of the disease even in the heterozygous form of the PC-2 protein. Although that variant retained the subcellular localization and C terminal–mediated protein interaction and
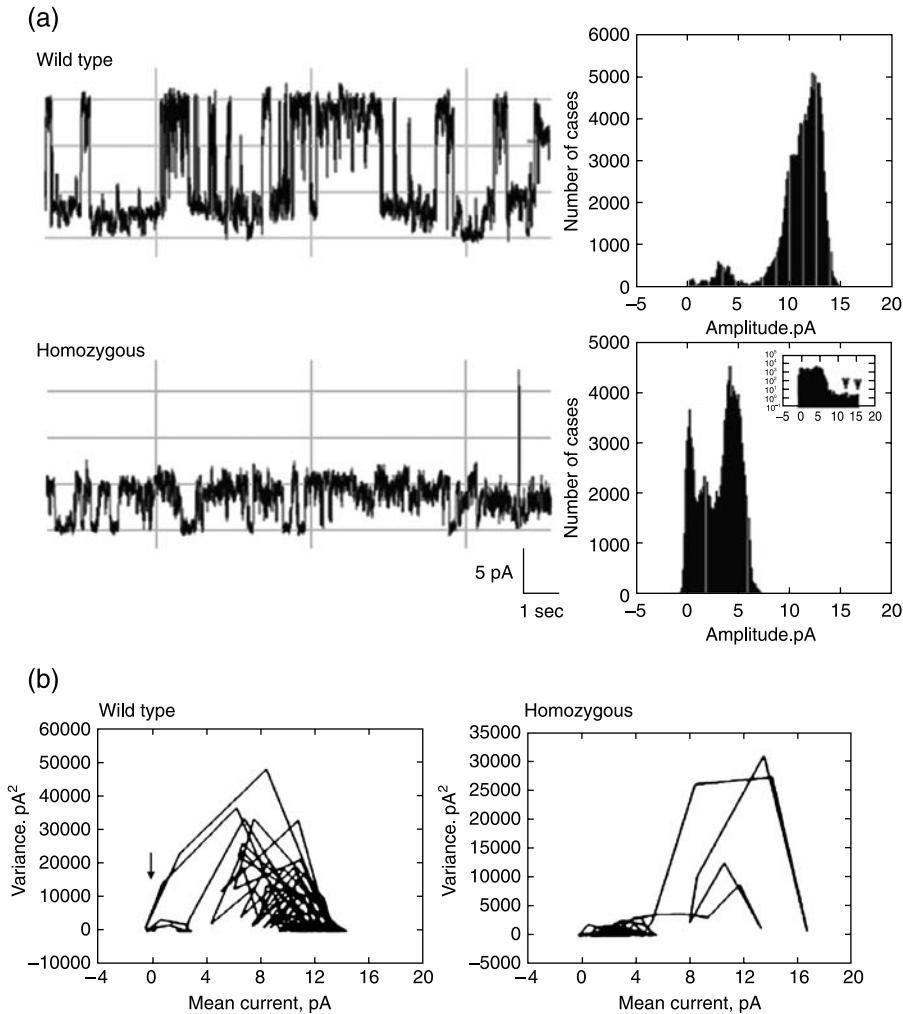
Figure 7 PC2 single channel currents in human PBMCs. (a) left: Single channel tracings of wild-type and homozygous mutant PC2. Tracings were obtained from excised inside-out patches in symmetrical K$^+$, with KCl in the bathing solution and K$^+$ aspartate in the pipette. Right: All-point histograms show the maximal conductance of the channel. Inset: Same histogram in a linear-log scale to indicate non frequent high conductance currents in the mutant PC2. Data are representative of seven control donors and four cells from a homozygous donor. (b) Mean vs. variance analysis showing the presence of subconductance states in PC2 channels from both control donors (left) and homozygous mutant (right). Both channels flicker to a high conductance state. The mutant PC2, however, displays only one such transition. Data are representative of four experiments for each group.

regulatory domains of wild-type protein the loss of channel function was sufficient to cause PKD. However our data regarding the reducing channel activities of the heterozygote led us to conclude that the amount of disruption of PC-2 channel is critical to the development of PKD disease. The patch clamp results indicate that both heterozygote and homozygote express a functional PC2 channel. However, this channel function displays, on average, a lower conductance (residence time of substrate, not a change in maximal single channel conductance) than in controls, which is reflected in a change in its kinetic properties as compared to the wild-type channel protein. One possible explanation for these results is dysfunctional channel assembly between wild-type and mutant PC2. Another not mutually exclusive possibility is the regulation by PC1, such as 'dysfunctional assembly' and/or regulation by PC1. It has been previously shown [38] that the location of mutations within the PKD2 gene influences clinical outcome. In the same study the authors revealed that mutations in exons 6 through 8 cause intermediate severity. The F482C is located in exon 6 therefore less severe aberration and PKD1 variant presence produce a more severe clinical phenotype of ADPKD disease (early onset of the disease) than the common PKD1 disease.

During ischaemic acute renal failure, which affects primarily the S3 segment of the proximal tubule, a pronounced up-regulation of polycystin-2 and a predominantly combined homogeneous and punctated cytoplasmic distribution in damaged cells has been previously observed [39]. In agreement with these data we found an overexpression of PC2 and a more diffuse cytoplasmic pattern in IMCD transfected cells with the mutant construct and perinuclear and cytoplasmic staining in skin biopsy which is different from ER membrane localization previously shown in ADPKD lymphoblastoid cells [40] as well as in kidney epithelial cells [41]. Additionally, it has been previously shown [42] that the level of polycystin-2 expression relative to that of polycystin-1 could influence the localization of polycystin-1 in mammalian cells. When this ratio is as high as it seems to be in our case,
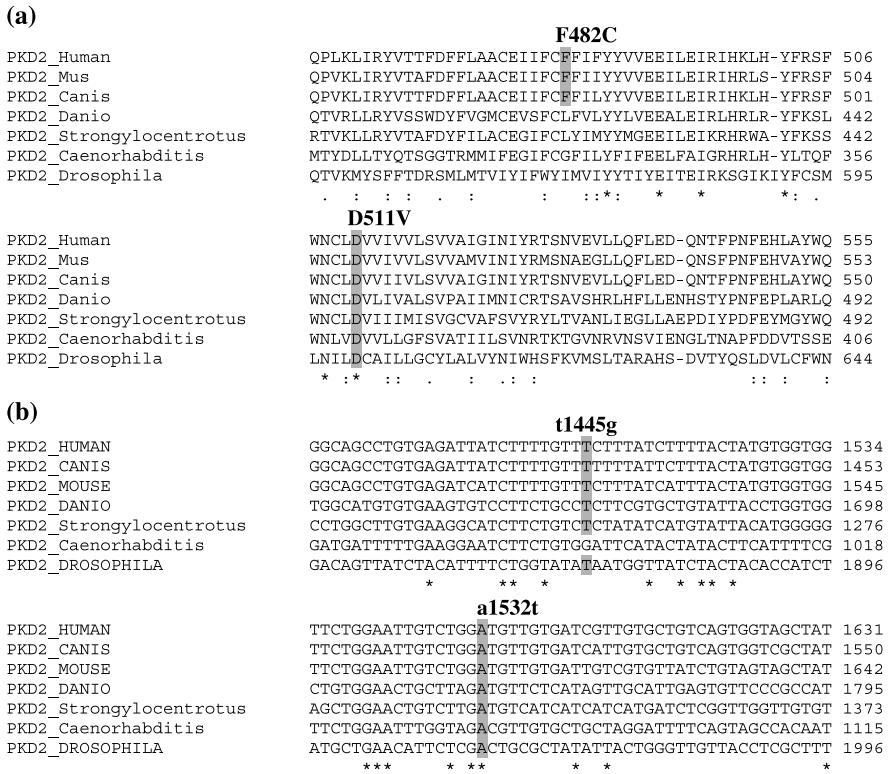
(a)

		F482C	
PKD2_Human	QPLKLIRYVTTFDFFLAACEIIFCFFIFYYVVEEILEIRIHKLH-YFRSF	506
PKD2_Mus	QPVKLIRYVTAFDFFLAACEIIFCFFIIYYVVEEILEIRIHRLS-YFRSF	504
PKD2_Canis	QPVKLIRYVTTFDFFLAACEIIFCFFILYYVVEEILEIRIHKLH-YFRSF	501
PKD2_Danio	QTVRLLRYVSSWDYFVGMCEVSFCLFVLYYLVEEALEIRLHRLR-YFKSL	442
PKD2_Strongylocentrotus	RTVKLLRYVTAFDYFILACEGIFCLYIMYYMGEEILEIKRHRWA-YFKSS	442
PKD2_Caenorhabditis	MTYDLLTYQTSGGTRMMIFEGIFCGFILYFIFEELFAIGRHRLH-YLTQF	356
PKD2_Drosophila	QTVKMYSFFTDRSMLMTVIYIFWYIMVIYYTIYEITEIRKSGIKIYFCSM	595
	. : :: . : : ::*: * * *: .

		D511V	
PKD2_Human	WNCLDVVIVVLSVVAIGINIYRTSNVEVLLQFLED-QNTFPNFEHLAYWQ	555
PKD2_Mus	WNCLDVVIVVLSVVAMVINIYRMSNAEGLLQFLED-QNSFPNFEHVAYWQ	553
PKD2_Canis	WNCLDVVIIVLSVVAIGINIYRTSNVEVLLQFLED-QNTFPNFEHLAYWQ	550
PKD2_Danio	WNCLDVLIVALSVPAIIMNICRTSAVSHRLHFLLENHSTYPNFEPLARLQ	492
PKD2_Strongylocentrotus	WNCLDVIIIMISVGCVAFSVYRYLTVANLIEGLLAEPDIYPDFEYMGYWQ	492
PKD2_Caenorhabditis	WNLVDVVLLGFSVATIILSVNRTKTGVNRVNSVIENGLTNAPFDDVTSSE	406
PKD2_Drosophila	LNILDCAILLGCYLALVYNIWHSFKVMSLTARAHS-DVTYQSLDVLCFWN	644
	* :* :: . : .: : :: : :

(b)

		t1445g	
PKD2_HUMAN	GGCAGCCTGTGAGATTATCTTTTGTTTCTTTATCTTTTACTATGTGGTGG	1534
PKD2_CANIS	GGCAGCCTGTGAGATTATCTTTTGTTTTTTTATTCTTTACTATGTGGTGG	1453
PKD2_MOUSE	GGCAGCCTGTGAGATCATCTTTTGTTTCTTTATCATTTACTATGTGGTGG	1545
PKD2_DANIO	TGGCATGTGTGAAGTGTCCTTCTGCCTCTTCGTGCTGTATTACCTGGTGG	1698
PKD2_Strongylocentrotus	CCTGGCTTGTGAAGGCATCTTCTGTCTCTATATCATGTATTACATGGGGG	1276
PKD2_Caenorhabditis	GATGATTTTTGAAGGAATCTTCTGTGGATTCATACTATACTTCATTTTCG	1018
PKD2_DROSOPHILA	GACAGTTATCTACATTTTCTGGTATATAATGGTTATCTACTACACCATCT	1896
	* ** * * * ** *

		a1532t	
PKD2_HUMAN	TTCTGGAATTGTCTGGATGTTGTGATCGTTGTGCTGTCAGTGGTAGCTAT	1631
PKD2_CANIS	TTCTGGAATTGTCTGGATGTTGTGATCATTGTGCTGTCAGTGGTCGCTAT	1550
PKD2_MOUSE	TTCTGGAATTGTCTGGATGTTGTGATTGTCGTGTTATCTGTAGTAGCTAT	1642
PKD2_DANIO	CTGTGGAACTGCTTAGATGTTCTCATAGTTGCATTGAGTGTTCCCGCCAT	1795
PKD2_Strongylocentrotus	AGCTGGAACTGTCTTGATGTCATCATCATCATGATCTCGGTTGGTTGTGT	1373
PKD2_Caenorhabditis	TTCTGGAATTTGGTAGACGTTGTGCTGCTAGGATTTTCAGTAGCCACAAT	1115
PKD2_DROSOPHILA	ATGCTGAACATTCTCGACTGCTGCGCTATATTACTGGGTTGTTACCTCGCTTT	1996
	*** * ** * * *

Figure 8 Alignment of PC2 sequences including the F482C and the D511V missense mutations. (a) Protein alignment. (b) Coding sequence alignment. The F482 is partially conserved among species compared with the D511, which is highly conserved.

polycystin-1 is found exclusively in the ER, where it colocalizes with PC-2. Similar expression pattern of both polycystins was observed in the patient's skin biopsy.

To our best of our knowledge, a living patient with ADPKD disease, homozygous for a novel PKD2 missense variant and heterozygous for a de novo splice-site mutation is reported hereby for the first time.

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