A missense mutation in PKD1 attenuates the severity of renal disease

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Mutations of *PKD1* and *PKD2* account for most cases of autosomal dominant polycystic kidney disease (ADPKD). Compared with PKD2, patients with PKD1 typically have more severe renal disease. Here, we report a follow-up study of a unique multigeneration family with bilineal ADPKD (NFL10) in which a PKD1 disease haplotype and a PKD2 (L736X) mutation co-segregated with 18 and 14 affected individuals, respectively. In our updated genotype–phenotype analysis of the family, we found that PKD1-affected individuals had uniformly mild renal disease similar to the PKD2-affected individuals. By sequencing all the exons and splice junctions of *PKD1*, we identified two missense mutations (Y528C and R1942H) from a PKD1-affected individual. Although both variants were predicted to be damaging to the mutant protein, only Y528C co-segregated with all of the PKD1-affected individuals in NFL10. Studies in MDCK cells stably expressing wild-type and mutant forms of PKD found that cell lines expressing the Y528C variant formed cysts in culture and displayed increased rates of growth and apoptosis. Thus, Y528C functions as a hypomorphic *PKD1* allele. These findings have important implications for pathogenic mechanisms and molecular diagnostics of ADPKD.

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Autosomal dominant polycystic kidney disease (ADPKD) is the most common hereditary kidney disease worldwide and accounts for ~5% of end-stage renal disease (ESRD) in North America.[1,2] It is characterized by focal and age-dependent development of renal cysts, leading to massive enlargement and distortion of the normal architecture of both kidneys and, ultimately, to ESRD in a majority of patients. Mutations of two genes, *PKD1* and *PKD2*, were thought to account for 85 and 15% of cases, respectively, in linkage-characterized European populations.[3] However, more recent population-based series suggest a higher prevalence of PKD2 ranging from 26 to 36%.[4] Disease progression of ADPKD is highly variable and in part due to a strong gene locus effect.[5–9] Adjusted for age and gender, PKD1 patients have larger kidneys and earlier onset of ESRD compared with PKD2 patients (mean age at ESRD: 53.4 vs. 72.7 years, respectively).[5–8] By contrast, a weak allelic effect (based on the 5′ vs. 3′ location of the germline mutations) on renal disease severity may be present for PKD1,[7] but not for PKD2.[8] In addition, marked intrafamilial renal disease variability in ADPKD suggests a modifier effect from genetic and environmental factors.[7–10]

PKD1 is a large gene consisting of 46 exons with an open reading frame of approximately 13 kb and is predicted to encode a protein of 4302 amino acids. Its entire 5′ region up to exon 33 has been duplicated six times on chromosome 16p, and the presence of these highly homologous pseudogenes has made genetic analysis of *PKD1* challenging.[1,2] Recent availability of protocols for long-range and locus-specific amplification of *PKD1* has enabled complete mutation screening of this complex gene.[11–14] By contrast, *PKD2* is a single-copy gene consisting of 15 exons with an open reading frame of approximately 3 kb and is predicted to encode a protein of 968 amino acids.[1,2] Recent studies of two large cohorts of patients with ADPKD have indicated that *PKD1* is a highly polymorphic gene.[13,14] On average, 10–13 polymorphic variants were identified in *PKD1* compared with ~1 in *PKD2*. Marked allelic heterogeneity is also evident for ADPKD, with over 400 different *PKD1* and ~100 different *PKD2* mutations reported to date that are thought

to be definitively or highly likely pathogenic (http://pkdb.mayo.edu/cgi-bin/mutations.cgi).[2,11–15] The majority of these mutations are unique and scattered throughout both genes. In general, protein-truncating (because of frame-shift deletion/insertion, nonsense changes, or splice defects) mutations, which are considered definitively pathogenic, have been identified in only 47–63% of patients.[12–14] By contrast, 'unclassified variants' (e.g., in-frame deletions and missense variants resulting in non-synonymous amino-acid substitutions) with pathogenic potential have been detected in an additional 26–37% of patients.[13–14] Although the clinical significance of the latter class of sequence variants remains to be defined, two recent studies suggest that certain missense *PKD1* mutations might function as hypomorphic alleles associated with mild or atypical renal disease.[16,17] Here, we provide further evidence to support this emerging concept.

In 2001, we reported a large multigeneration family (NFL10) with bilineal ADPKD in which a PKD1 disease haplotype and a PKD2 (L736X) mutation co-segregated with 18 and 14 affected individuals, respectively (Figure 1).[18] After excluding the PKD2-affected subjects in this family, we performed linkage analysis and obtained a highly significant LOD (logarithm (base 10) of odds) score of ~5.0 at KG8 (an intragenic marker within *PKD1*), strongly suggesting that the disease in the remaining affected members was due to PKD1. Consistent with our linkage results, we found perfect segregation of a putative PKD1 disease haplotype (D16S521-HBAP1-KG8-D16S665-D16S291-D16S2618: 3-4-1-7-8-5) in the 16 affected members who did not have the *PKD2* mutation. Two additional subjects with trans-heterozygous mutations of both genes had more severe renal disease compared with subjects affected with only PKD1 or PKD2 alone, providing for the first time evidence for genetic interaction in ADPKD. Interestingly, the renal disease of the PKD1-affected subjects in NFL10 appeared very mild and was indistinguishable from those affected with PKD2.

RESULTS

Genotype–phenotype correlation

In the current report, we have updated the most recent information on the renal function of the affected subjects from NFL10 with an additional follow-up of 7.5 years. Confirming our previous impression, we found that PKD1-affected subjects continued to have mild disease that was indistinguishable from that of PKD2-affected subjects (Figure 2a).

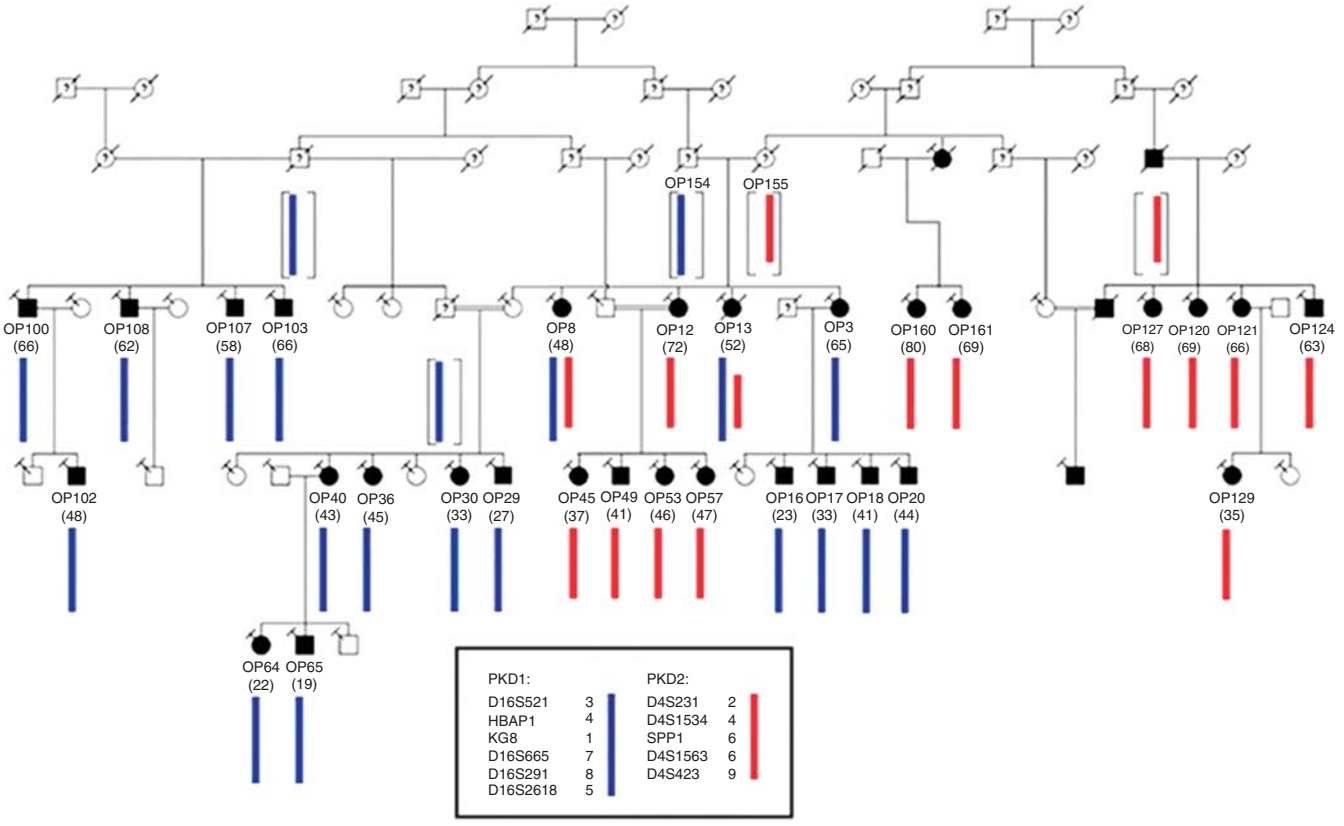

Figure 1 | Disease segregation pattern in NFL10. Fourteen affected individuals carrying the PKD2 (L736X) mutation co-segregated with the PKD2 haplotype (D4S231-D4S1534-SPP1-D4S1563-D4S423: 2-4-6-6-9; denoted by the red bar), whereas 16 affected individuals without the PKD2 mutation co-segregated with the putative PKD1 disease haplotype (D16S521-HBAP1-KG8-D16S665-D16S291-D16S2618: 3-4-1-7-8-5; denoted by the blue bar). Two members (OP8 and OP13) of this family affected with autosomal dominant polycystic kidney disease carried both mutations. The number in parentheses denotes the age at the most recent clinical assessment. Double horizontal line denotes marriage between two related individuals.

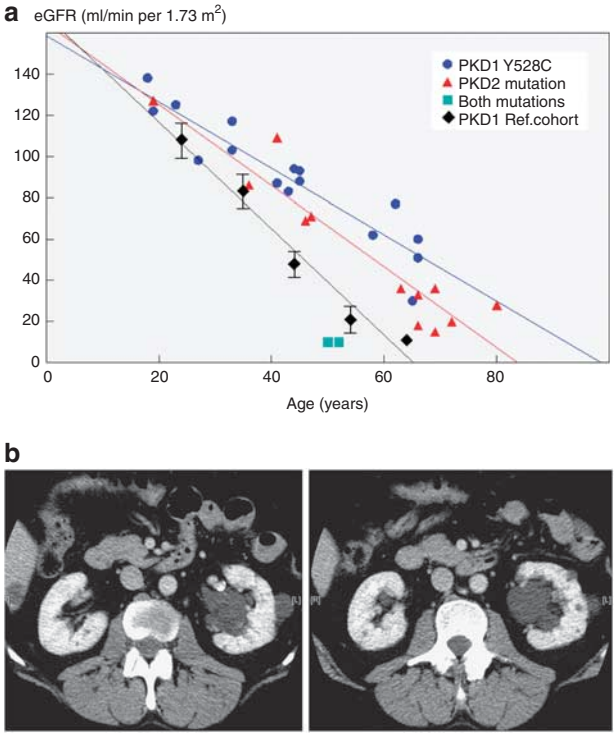

Figure 2 | Genotype–phenotype correlation in NFL10. (**a**) Renal disease severity in subjects affected with PKD1 (Y528C) and PKD2 was very similar in this pedigree. Linear regression analysis showed that the slope of estimated glomerular filtration rate (eGFR) by age did not differ between affected subjects with the two gene types (PKD1: −1.61 (95% confidence interval (CI): −1.25 to −1.97); PKD2: −1.94 (95% CI: −1.45 to −2.44)). However, two subjects affected with both PKD1 and PKD2 had more severe renal disease compared with subjects affected with only PKD1 or PKD2 alone. For comparison, the mean eGFR (95% CI) from a large cohort of PKD1 patients is also presented according to the age strata of 20–29 ($n = 44$), 30–39 ($n = 92$), 40–49 ($n = 132$), 50–59 ($n = 79$), and 60–69 ($n = 30$) at ages 25, 35, 45, 55, and 65 years, respectively. The CI was not shown in the last age group, as the eGFR was not normally distributed. (**b**) The computed tomography scan performed on OP103 at age 60 years showed only mildly enlarged kidneys with numerous small cysts in the left kidney and multiple small cysts in the right kidney. A large left peripelvic renal cyst and a single liver cyst were also noted.

Indeed, the linear regression analysis showed that the slope of estimated glomerular filtration rate (eGFR) by age did not differ among the affected subjects with the two gene types (−1.61 (95% confidence interval: −1.25 to −1.97) for PKD1 vs. −1.94 (95% confidence interval: −1.45 to −2.44) for PKD2). Moreover, compared with a large cohort of PKD1 patients, those individuals with Y528C clearly had better preserved renal function. Four PKD1-affected members were over 60 years of age at their last clinical follow-up and none had developed ESRD. Renal imaging studies were available for two of these older PKD1-affected subjects. A computed tomography scan performed on OP103 at age 60 years showed normal-sized kidneys (right $6.9 \times 6.5 \times 12$ cm; and left $8.1 \times 7.6 \times 11.1$ cm) with innumerable subcentimeter-sized small cysts located bilaterally (Figure 2b). Similarly, an ultrasound performed on OP100 at age 59 years showed normal-sized kidneys (right 11 cm and left 12 cm in length) with numerous small renal cysts located bilaterally and three liver cysts. These extremely mild findings of renal cystic disease are highly atypical of PKD1.

Identification of a pathogenic *PKD1* mutation

To identify the pathogenic *PKD1* mutation in NFL10, bidirectional sequencing of all the exons and splice junctions of *PKD1* was performed in the genomic DNA of OP100, and 26 sequence variants were detected (Supplementary Table S1 online). Most of the sequence variants were known or predicted polymorphisms, except for two novel missense variants, Y528C and R1942H, both of which were predicted to be damaging by PolyPhen (http://genetics.bwh.harvard.edu/pph/). The PSIC profile score difference was 2.4 for Y528C and 1.8 for R1942H. However, only Y528C (c.1583A>G) co-segregated with all 18 PKD1-affected individuals (including the two trans-heterozygotes, OP8 and OP13). The Y528C variant affects an amino-acid residue in the C-type lectin domain of polycystin-1 (PC1) that is highly conserved across multiple species (Figure 3) and moderately conserved across non-PC1 proteins with the same domain (Supplementary Figure S1 online).

In vitro analysis of Y528C

To establish the pathogenicity of Y528C, we performed *in vitro* functional studies in stable MDCK cell lines expressing tetracycline-inducible wild-type and mutant (Y528C) forms of PC1. We found that both wild-type and mutant PC1 were equally expressed and that the Y528C mutation did not disrupt the normal cleavage of PC1 at the GPS site (Supplementary Figure S2 online).[14] We have previously established that MDCK cells form cysts when grown in three-dimensional collagen cultures. Expression of PC1 in these cells is sufficient to induce tubulogenesis along with reduced growth rates and resistance to apoptosis.[19] We found that MDCK cell lines expressing Y528C continued to form cysts in culture, similar to what has been demonstrated for a fully penetrant mutant control.[20] In addition, Y528C-expressing cell lines demonstrated increased rates of growth and apoptosis, similar to what was observed in the empty vector control (Figure 4). One of the limitations of this *in vitro* assay is its inability to discriminate between complete and partial phenotypes. However, taken together with the uniformly mild renal phenotype observed in individuals harboring Y528C, the above data strongly suggest that this variant is the pathogenic *PKD1* mutation in NFL10 and that it functions as a hypomorphic allele *in vivo*.

DISCUSSION

Three conclusions can be drawn from our study on NFL10. First, the observation that PKD1-affected individuals consistently displayed attenuated renal disease argues in favor of an allelic effect associated with the Y528C mutation, rather than a modifier effect.[9,10] Second, our study is consistent with

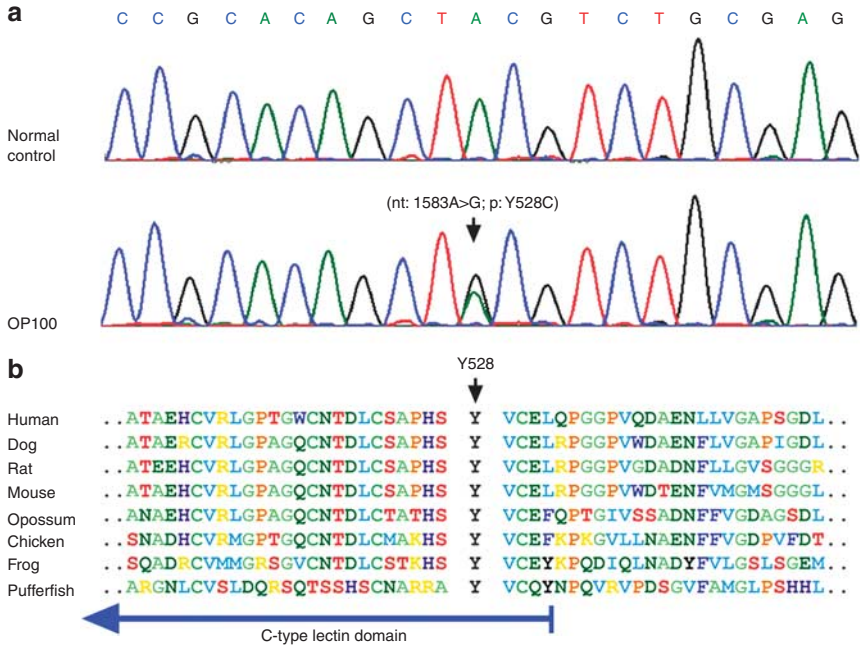

Figure 3 | Co-segregation of Y528C with PKD1 disease haplotype in NFL10. (a) A highly conserved *PKD1* missense (nt: A1583G; p: Y528C) mutation co-segregated with all 18 affected subjects with the putative PKD1 disease haplotype. (**b**) Y528C affects an amino-acid residue in the C-type lectin domain of polycystin-1, which is highly conserved across multiple species including the pufferfish, fugu.

the recent concept that a critical cellular polycystin threshold is involved in cystogenesis. Previous studies have provided strong evidence in support of a cellular recessive model in which homozygous inactivation of *PKD1* or *PKD2* by germline and somatic mutations could trigger cystogenesis in ADPKD.[21–23] However, recent studies on homozygous *Pkd1* knockout mice with aberrant mRNA splicing indicate that complete biallelic inactivation may not be absolutely required, as renal cystogenesis can be triggered by normal PC1 levels below a 10–20% threshold.[24,25] Within the 'two-hit model' framework, data from our current and two recent studies[16,17] are consistent with the notion of a critical cellular polycystin threshold for cystogenesis.[24,25] Third, two recent molecular diagnostic surveys of patients with ADPKD have identified protein-truncating mutations in only 47–63% of the cases, with 26–37% of additional cases reported to have 'unclassified variants' (in-frame deletions and non-synonymous sequence variants) with pathogenic potential.[13,14] From the latter patient cohort, it is likely that more cases of hypomorphic *PKD1* alleles will be increasingly recognized. An outstanding question of major clinical importance is what proportion of these 'unclassified variants' function as hypomorphic alleles and therefore might be associated with mild renal disease. This can only be answered by systematic genetic–phenotype analyses involving a large number of cases. In the meantime, unambiguous demonstration of hypomorphic *PKD1* alleles from recent clinical studies implies that mild renal disease is no longer restricted to patients with PKD2 (refs 5 and 8) and further extends the spectrum of renal disease variability in ADPKD.

MATERIALS AND METHODS

Study subjects and clinical assessment

We have previously ascertained the pedigree structure of NFL10 consisting of at least 130 members over six generations. Forty-four at-risk individuals and four spouses who gave informed consent were studied. All the at-risk individuals and spouses were screened with at least one abdominal ultrasound. All the scans were performed under the supervision of one ultrasonographer and interpreted by a radiologist (Dr Benvon Cramer, Department of Radiology, Memorial University, St John's, Newfoundland) without any knowledge of the clinical status of the subjects using the Ravine criteria.[26] The research protocols used for this study were approved by the Human Subject Review Committees of the University of Toronto and Memorial University in Newfoundland. In this update, we repeated the serum creatinine measurements in 30 of 32 affected subjects and reassessed their renal function as eGFR using the MDRD formula.[27] Two affected subjects with ESRD were assigned a default eGFR of 10 ml/min. For comparison purpose, we used the data from a large cohort of PKD1 patients (excluding NFL10) that we had studied previously[28] to generate mean eGFR (95% confidence interval) by each decade plotted at the midpoints of the following age groups: 20–29 ($n = 44$), 30–39 ($n = 92$), 40–49 ($n = 132$), 50–59 ($n = 79$), and 60–69 ($n = 30$). We did not present any confidence interval for the last age group, as the data were not normally distributed.

Mutation screening

Mutation screening of *PKD1* and *PKD2* was performed for OP100 using a commercial diagnostic service (Athena Diagnostics, Worcester, MA; http://www.athenadiagnostics.com/).[13] Genomic DNA was used as a template for specific long-range PCR amplification of eight segments encompassing the entire *PKD1* duplicated region. The long-range PCR products served as templates for 43

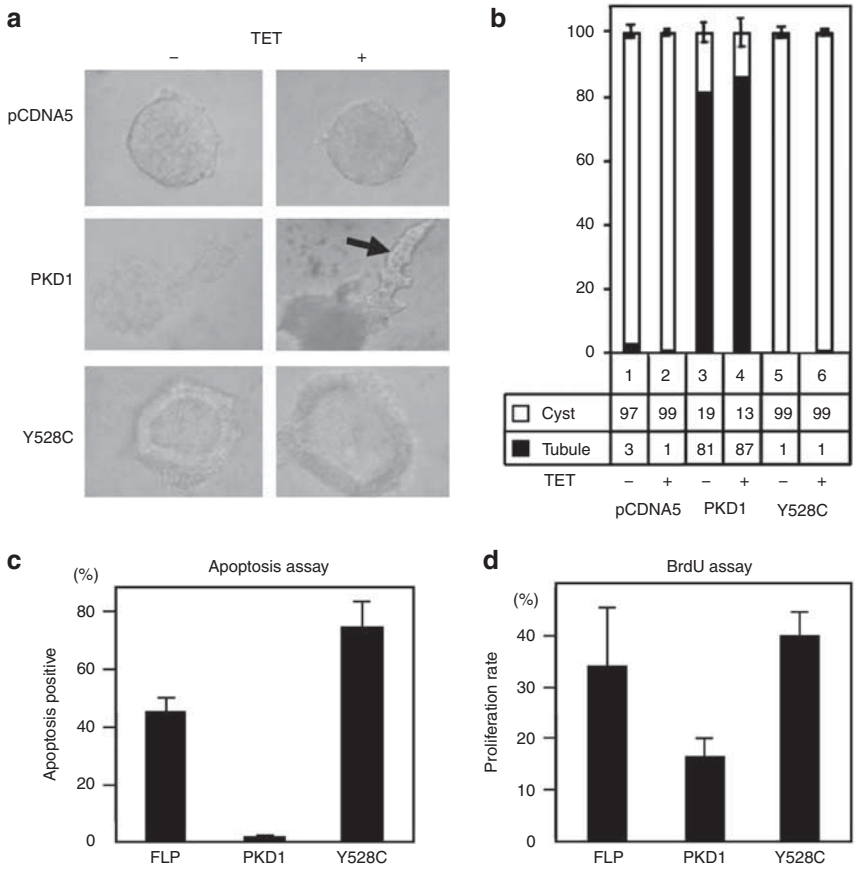

Figure 4 | *In vitro* functional analysis of stable MDCK cell lines expressing wild-type and mutant Y528C forms of PKD1. (**a**) Representative example of the tubulogenesis assay in the absence (−) or presence (+) of tetracycline (TET). Almost 100% of the vector control cells form cysts, whereas PKD1-expressing cells form tubules regardless of induction with tetracycline. On the basis of western analysis this is explained by leaky polycystin-1 expression, which occurs even in the absence of tetracycline. The Y528C cell line exhibits 'mutant' behavior and forms cysts. The arrow indicates a tubule formed by a PKD1-expressing cell. (**b**) The percentage of cells forming cysts or tubules was assayed 2 weeks after culture and was tabulated for three independent clonal isolates. (**c**) Apoptosis assay by terminal deoxytransferase uridine triphosphate nick end labeling staining. The vector control (FLP) consistently exhibits an apoptotic rate of ~45%. The apoptotic rate drops markedly to less than 10% in PKD1-expressing cell lines. The Y528C-expressing cell line shows a relatively high apoptotic rate that is not statistically different from vector control. (**d**) Cell proliferation by 5-bromodeoxyuridine (BrdU) incorporation. PKD1-expressing cells had a lower rate of proliferation when compared with the vector control. Growth rates for Y528C resembled the control.

nested PCRs, whereas the unique region of *PKD1* and the entire PKD2 were amplified from genomic DNA in 28 additional PCRs. All 71 PCR products were bidirectionally sequenced, including the coding regions and exon–intron splice junctions of both genes. To confirm the segregation of disease-associated variants, long-range and nested PCR products for exons 7 (for Y528C) and 15 (for R1942H) were amplified from genomic DNA using published primers and conditions.[14] The nested PCR products were then sequenced.

Predicting deleterious missense mutations

We used the software PolyPhen to evaluate the functional impact of a number of unclassified missense variants that alter a single amino-acid residue. Upon entry of the protein ID, and the wild-type and mutant amino-acid variants, PolyPhen conducts a comprehensive search to identify all the homologous protein sequences. On the basis of alignment of these homologous protein sequences, PolyPhen computes profile scores for both the allelic variants.[28] Profile scores are logarithmic ratios of the likelihood of a given amino acid occurring at a particular site to the likelihood of this amino acid occurring at any site (background frequency). A variant is predicted to be damaging if the absolute difference between the profile scores of two amino-acid variants is >1.7.[29]

In vitro functional analysis

The wild-type PKD1 construct was derived from pCI-PKD1-Flag.[19] The Y528C mutant was generated from this base construct using site-directed mutagenesis (Stratagene, La Jolla, CA). Stable MDCK cell lines expressing flag-tagged wild-type or mutant (Y528C) PC1 were generated using an Flp-In tetracycline inducible system (Invitrogen, Carlsbad, CA). A vector-only cell line was used as a control. PC1 expression was induced with tetracycline (TET) and the cells were cultured with selection agents hydromycin (100 μg/ml) and blasticidin (5 μg/ml). The tubulogenesis assay was performed using the methods reported by Boletta *et al.*[19] Cells forming cysts or tubules were assayed 2 weeks after culture and tabulated for three independent clonal isolates. After the cells were serum starved for 72 h, apoptosis was assessed by terminal deoxytransferase uridine triphosphate nick end labeling staining and cell proliferation was

assessed by 5-bromodeoxyuridine incorporation.[19] All the assays were performed in triplicate. Antibodies used for immunodetection of PC1 have been previously described.[20]

Statistical analysis

Linear regression analysis on the rate of loss of eGFR on the basis of age in subjects affected with PKD1 or PKD2 was performed using the equation fitting suite of the software SlideWrite Plus version 7.01 (Advanced Graphics Software, Santa Fe, CA).

DISCLOSURE

All the authors declared no competing interests.

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SUPPLEMENTARY MATERIAL

Table S1. PKD1 and PKD2 gene variants identified from OP100.
Figure S1. Alignment and comparison of 44 non-redundant full-length sequences from proteins with C-type lectin domain by Pfam (http://pfam.sanger.ac.uk/) showing moderate conservation (score 4/10) at residue Y528 (boxed in red).
Figure S2. Y528C did not alter polycystin cleavage at the GPS site.
Supplementary material is linked to the online version of the paper at http://www.nature.com/ki

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