



Published in final edited form as:

Mol Genet Metab. 2007 ; 92(1-2): 160–167.

Evaluating the Clinical Utility of a Molecular Genetic Test for Polycystic Kidney Disease

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Abstract

Autosomal Dominant Polycystic Kidney Disease (ADPKD) is estimated to affect 1/600–1/1000 individuals worldwide. The disease is characterized by age dependent renal cyst formation that results in kidney failure during adulthood. Although ultrasound imaging may be an adequate diagnostic tool in at risk individuals older than 30, this modality may not be sufficiently sensitive in younger individuals or for those from PKD2 families who have milder disease. DNA based assays may be indicated in certain clinical situations where imaging cannot provide a definitive clinical diagnosis. The goal of this study was to evaluate the utility of direct DNA analysis in a test sample of 82 individuals who were judged to have polycystic kidney disease by standard clinical criteria. The samples were analyzed using a commercially available assay that employs sequencing of both genes responsible for the disorder. Definite disease causing mutations were identified in 34 (~42%) study participants. An additional 30 (~37%) subjects had either in frame insertions/deletions, non-canonical splice site alterations or a combination of missense changes that were also judged likely to be pathogenic. We noted striking sequence variability in the *PKD1* gene, with a mean of 13.1 variants per participant (range 0–60). Our results and analysis highlight the complexity of assessing the pathogenicity of missense variants particularly when individuals have multiple amino acid substitutions. We conclude that a significant fraction of ADPKD mutations are caused by amino acid substitutions that need to be interpreted carefully when utilized in clinical decision-making.

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Conflict of Interest Disclosure:

Under a licensing agreement between Athena Laboratories and the Johns Hopkins University, Dr. Watnick and Dr. Germino are entitled to a share of royalty received by the University on sales of products described in this article. They have elected to donate their share of the royalty to the Polycystic Kidney Disease Research Foundation. The terms of this arrangement are being managed by the Johns Hopkins University in accordance with its conflict of interest policies.

Keywords

Autosomal dominant polycystic kidney disease; ADPKD; mutation analysis; PKDx[®]; PKD1; PKD2; DNA testing

Introduction

Autosomal dominant polycystic kidney disease (ADPKD [MIM 173900]) is one of the most common inherited disorders in humans, affecting ~1/600–1/1000 individuals [1]. The disease is characterized by age dependent growth of renal cysts such that end stage renal disease (ESRD) typically ensues during mid adulthood. Approximately 85% of ADPKD cases are caused by mutations in the *PKD1* gene [MIM 601313], which is located on chromosome 16, while the remaining cases are due to mutations in *PKD2* [MIM 173910] located on chromosome 4 [2–7].

Renal ultrasound is a sensitive method of diagnosing ADPKD in those individuals older than 30 years of age who are at risk for inheriting *PKD1* mutations. Since cyst formation is an age dependent process, the false negative rate of ultrasound is higher in younger individuals or in those with *PKD2* mutations, which are associated with later onset disease [8,9]. There are a number of clinical scenarios in which DNA based testing for ADPKD might be indicated. Ultrasound imaging might not provide a sufficiently certain diagnosis in at risk individuals younger than 30 years of age who wish to donate a kidney to a relative with renal failure. In addition, clinicians may encounter patients with atypical cystic disease in whom the diagnosis may not be obvious. Finally, new therapies for ADPKD are on the horizon and there is evidence to suggest that these may be most effective if initiated early in the disease course, perhaps even before cystic disease is apparent [10–12].

Linkage analysis for ADPKD has been commercially available for some time, but requires the participation of at least two and preferably more affected family members. In cases where family members are not available, direct DNA analysis offers the possibility of establishing a molecular diagnosis. Direct DNA testing for ADPKD has posed a unique set of challenges. *PKD1* analysis in particular has been complicated because the 5' portion of the gene (exons 1–34) is replicated in at least 5 highly homologous copies (<2% divergence) elsewhere on chromosome 16 [6]. Several groups have now been able to analyze the *bona fide PKD1* sequence by using gene-specific primers to amplify large products that can then be screened via nested PCR using direct sequencing [13–24] or other techniques such as denaturing high-performance liquid chromatography (DHPLC) [25–28].

In the current study, we report an analysis of direct sequencing results for both *PKD1* and *PKD2* in a cohort of 82 individuals with the clinical features of polycystic renal disease (PKDx[®], Athena Diagnostics, Inc). Since a major indication for direct DNA testing is the absence of family members for linkage analysis, we chose to study one individual from each family. We identified truncating mutations in ~42% (N=34) of the participants. An additional 30 (~37%) study subjects had either in frame insertions/deletions, non canonical splice site alterations or a combination of missense changes that were judged likely to be pathogenic. Direct sequencing is a sensitive method of detecting DNA variability in *PKD1* and *PKD2*, but given the significant proportion of non-truncating mutations, the need for strict interpretation is essential if the results are to be used in clinical decision-making. To our knowledge, this is the first report of direct sequencing of the entire coding regions of both *PKD1* and *PKD2*.

Materials and Methods

Patient Recruitment and Clinical Evaluation

Eighty-two unrelated ADPKD patients were recruited from outpatient nephrology clinics at the Johns Hopkins Hospital and the University of Toronto. Patients were seen at various stages of the disease process. Institutional review boards at the Johns Hopkins School of Medicine and the University of Toronto approved the study. Informed consent was obtained from each participant. We based the diagnosis of ADPKD on the Ravine ultrasound criteria [8]. A detailed medical history and a coded blood sample were obtained at the time of entry into the study. Blood samples were sent to Athena Diagnostics, Inc. for mutation analysis using the same methodology as for commercially obtained samples (<http://www.athenadiagnostics.com/content/test-catalog/find-test/service-detail/q/id/249>). In most cases, routine laboratory data was obtained as part of the standard medical evaluation.

Mutation Analysis

Sequence analysis was performed using previously described methods optimized at Athena [13–15,19,21,29,30]. Briefly, genomic DNA was derived from whole blood using Puregene[®] DNA extraction kit (Gentra Systems, Inc.) and used as template for specific long-range PCR amplification of 8 segments encompassing the entire *PKD1* duplicated region. The long-range PCR products served as template for 43 nested PCR reactions while the unique region of the *PKD1* gene and the entire *PKD2* gene were amplified from genomic DNA in 28 additional gene segments. All 71 PCR products were bi-directionally sequenced including the coding regions and exon-intron splice junctions of both *PKD1* and *PKD2*.

Analysis of Normal Samples

The normal population was selected from anonymized samples, older than 65, submitted to Athena for ataxia testing. PCR products from a minimum of 171 individuals were sequenced to determine the frequency of certain common variants in either *PKD1* or *PKD2*. Complete DNA analysis was not performed for these samples.

Cleavage Assay

Missense variants were generated using the QuickChange Site-Directed Mutagenesis Kit (Stratagene). The full-length, wild type *PKD1* cDNA, four additional constructs (Q3016R, F3064L, F2853S, E2771K) and transfection methods have been previously described [31, 32]. After transfection, the cells were lysed and lysates were immunoprecipitated using ANTI-FLAG[®] M2 beads (SIGMA) and resolved on NuPAGE[®] 3–8% Tris-Acetate Gels (Invitrogen). The products were electro-blotted onto an Immobilon[™] transfer membrane (MILLIPORE) and probed with α -Leucine-rich-repeat (LRR) and α -C-terminus (CT) antibodies for polycystin-1 (PC1) [32,33].

Web Resources

The following GenBank sequences (<http://www.ncbi.nlm.nih.gov/Web/Genbank/>) served as reference files: L39891 for *PKD1* genomic nucleotide position, L33233 for *PKD1* cDNA position, AAC37576 for *PKD1* amino acid position, V50928 for *PKD2* genomic nucleotide position, NM000297 for *PKD2* cDNA position and NP00288 for *PKD2* amino acid position. Other Web Resources: Online Mendelian Inheritance in Man (OMIM), <http://www.ncbi.nlm.nih.gov/Omim/>; Splice Site Prediction by Neural Network (SSPNN); <http://fruitfly.org/seqtools/splice.html> [34]; Automated Splice Site Analyses (ASSA), <https://splice.cmh.edu/>[35,36]; Simple Modular Architecture research tool (SMART), <http://smart.embl.de/>; Pfam, <http://www.sanger.ac.uk/Software/Pfam/>. MDRD equation: <http://nephron.com/cgi-bin/MDRDSI.cgi>.

Results

Patient Characteristics

We selected an outpatient cohort that was judged to have polycystic kidney disease based on standard ultrasound criteria (Table 1) [8].

Classification of Sequence Alterations

DNA sequencing results fell into three classes (Table 2). Class I tests were defined as those that had definitive pathogenic sequence variants, such as stop codons, frameshifts or canonical splice site alterations. Class II tests consisted of those demonstrating in frame deletion/insertions, non-canonical splice site mutations, or amino acid substitutions that were judged likely to be pathogenic based on the application of various algorithms. Class III tests included those where no pathogenic changes could be confirmed. The location of Class I and Class II variants are summarized in Figure 1.

Class I Tests

Thirty-four study participants (~42%) were found to have Class I variants (Table 2 and Supp Table 1 and 2). Twenty-four mutations occurred in *PKD1* (~29% of total sample) and ten in *PKD2* (~12% of total sample).

Class II Tests

Thirty study participants had Class II variants (Table 2 and Supp Tables 2–4). Participants with class II tests had a positive family history in 77% of cases, similar to 79% for Class I.

We detected a total of 7 unique *in-frame* deletions (6 *PKD1* and 1 *PKD2*) and 1 unique *in-frame* insertion (*PKD2*) in the study population (Supp Table 3). In each instance the in-frame change affected one or more residues that were fully or highly conserved between the *Fugu rubripes* and *Mus musculus* polycystin proteins. It seems likely that most if not all of these changes are pathogenic but we cannot exclude the possibility that a functional protein might be synthesized from these alleles.

There were 10 individuals with no other truncating PKD mutations who had unique intronic variants. Two of these sequence alterations (*PKD1*: IVS24+5 G>C in JHU573 and JHU595; *PKD2*: IVS8+5 G>A in JHU105) occurred at the 5th base pair from a splice-donor site, which is highly conserved as a guanine in 84% of cases [37]. These variants as well as two others were predicted to result in improper splicing by both the ASSA and SSPNN programs (Supp Table 2). In addition, IVS37-10C>A (JHU 604), was previously reported to segregate with ADPKD in a European family [38]. Although these intronic variants are likely to represent splicing mutations, aberrant splicing could not be confirmed at the RNA level using this DNA based assay.

Most of the remaining participants (N=29) had a combination of amino acid substitutions, primarily in *PKD1* (Supp Table 4). We used three major criteria to judge the pathogenicity of each missense variant. We examined the conservation of the altered residue between human polycystin-1 and both the *Fugu* and mouse proteins. In addition, we assigned a pathogenicity score for each missense variant using the matrix of Miller and Kumar [39], which defines the relative likelihood that a missense change represents a pathogenic alteration versus a polymorphism. This algorithm was developed by using interspecies sequence comparisons coupled with Grantham's chemical difference matrix to determine the common attributes of amino acid replacement mutations across 7 other disease genes. Other investigators have also used this strategy to assist in characterizing amino acid substitutions [40]. Finally, we reviewed the literature to determine whether any of the variants had been reported by others to occur in

normals. Our analysis of individual amino acid substitutions is summarized in Supp Table 4. We found that 17 participants had at least one variant that was likely to be pathogenic.

Several missense changes were predicted to disrupt key structural determinants of PKD1 (Figures 1 and 2). Three participants had variants that had been previously shown to disrupt polycystin-1 cleavage, a property that is critical for the protein's function [32]. We suspected that other amino acid substitutions near the polycystin-1 GPS site might also disrupt cleavage. In order to test this, we generated full-length mutant constructs and expressed them in HEK293 cells. We confirmed that E2771K, Q3016R and F2853S disrupt cleavage, as do three of these additional missense changes, R2643C, R2767C and L2619P (Figure 3).

There were several recurrent *PKD1* variants (R2200C, Q739R, G2814R, Q2182R) that had a higher pathogenic potential by the Miller/Kumar matrix [39] but which were also present in individuals with chain terminating mutations (Supp Tables 4 and 5). For example, R2200C was present in four participants and one (JHU111) also had a *PKD1* frame shift. This association suggested that R2200C might represent a polymorphism. We sequenced 342 normal chromosomes to examine a subset of these missense changes and identified R2200C in a small (1.4%) fraction but greater than the polymorphism threshold of 1%. Likewise Q739R (this study, 6.4%) and G2814R (Rossetti, *et al* ~0.9%) have also been reported in a small percentage of the unaffected population and are or may be polymorphisms, respectively [16, 26].

Five participants, JHU602 (N=2), JHU100 (N=3), JHU588 (N=2), JHU411 (N=2) and JHU114 (N=2), had more than one *PKD1* amino acid variant that met the criteria for pathogenicity (Supp Table 4). These observations raise the possibility that a combination of missense changes in *cis* might cooperatively result in a diminished level of functional protein [41].

In contrast with *PKD1*, there were only two *PKD2* amino acid substitutions and both were predicted to be polymorphisms (Supp Table 4). M800L (JHU559) did not segregate with disease in a *PKD2* family [42]. A second *PKD2* substitution, A190T, was identified in 3.2% of normal chromosomes.

Class III Tests

After conducting the analysis described above, there remained 18 subjects who lacked a definitive pathogenic sequence alteration (Individuals without shaded variants not underlined in Supp Table 4). Of these, 9 had a clear family history of ADPKD. All of the remainder (N=9) had enlarged cystic kidneys.

There are several reasons why we might have failed to identify a pathogenic change in this subset of individuals. Mutational events in individuals with class III tests could involve introns or other regulatory regions that were not assayed by the methodology that was used. Direct sequencing might also miss deletions or duplications, which would appear as an area of homozygous normal sequence. Alternatively, our stringent criteria may have identified some missense changes as benign when they may in fact be pathogenic. For example JHU617, with an extensive family history of ADPKD, was found to have a unique leucine to valine change in PKD repeat 4 that was judged more likely to be a polymorphism by the matrix of Miller/Kumar [39]. Nevertheless, this change does disrupt the structure of PKD repeat 4 and could be pathogenic (Figure 2). In addition, as reported by Reynolds, missense variants may unexpectedly activate cryptic splice sites, thereby reducing the level of normal transcript [43].

Polymorphisms, Private Normal Variants And Variability In PKD Genes

There was an impressive degree of sequence variability, particularly in the *PKD1* gene. In addition to the sequence alterations described in Supp Tables 1–5, we detected a large number of polymorphisms and private, non-pathogenic variants (see Supp Table 6). The density of variants was noted to be dramatically higher in *PKD1* as compared with *PKD2*. On average, the 82 study participants had 13.1 *PKD1* sequence variants per patient (range 0 to 60, Supp Tables 3–5). In contrast, there was an average of only 1.1 *PKD2* variants per patient (range of 0–3).

Discussion

We anticipate that there will continue to be a need for molecular diagnosis among individuals from ADPKD families. DNA testing may be relevant in a variety of clinical circumstances including the evaluation of living donors from ADPKD families, early detection for treatment with new therapeutic agents and in individuals with atypical clinical presentations where a diagnosis of ADPKD is in question. In situations where ultrasound is not sufficiently sensitive and where family members are unavailable for linkage studies, direct DNA testing has the potential to provide genetic information to an isolated proband. Direct sequencing of *PKD1* and *PKD2* forms of the basis for the analysis in this study. We have evaluated the utility of this test in 82 individuals with a clinical diagnosis of ADPKD.

We found that 42% of the study participants (N=34) had a definitive pathogenic mutation predicted to result in premature truncation of either PKD protein (Class I). The remaining study subjects had in frame deletions/insertions, amino acid substitutions or intronic changes requiring further analysis to assess pathogenicity (Class II). We chose not to study the segregation of these variants since the lack of available family members is an important indication for the use of direct sequencing. Instead we utilized several *in silico* approaches to determine which variants were more likely to be disease-related. This extended analysis allowed us to conclude that 30 individuals with Class II test results had at least one variant that was deemed likely to be pathogenic. Further evidence of disease relatedness for a subset of variants was provided by *in vitro* functional studies demonstrating that six of the missense amino acid substitutions resulted in loss of polycystin-1 cleavage [32]. If one combines Class I and Class II tests, mutations were detected in approximately 78% (N=64) of the study population.

Only one other group has published a study similar to ours. Rossetti et al used DHPLC to screen *PKD1* and *PKD2* in 45 genetically uncharacterized individuals with clinical ADPKD [26]. In their research cohort, 34 (76%) subjects had class I or class II tests. This is not statistically different from our results (78%, $p \leq 1.0$, Chi-square test). The differences in the incidence of truncating mutations between our cohort and Rossetti's may be related to characteristics of the study population. Our cohort was not identified on the basis of ESRD so we may have selected for a group with a milder phenotype and hence a lower prevalence of truncating mutations.

There are several reports that have employed a combination of screening methods including protein truncation, DHPLC and selective sequencing to identify mutations in portions of *PKD1* or *PKD2* [13–19,21–27,29]. We note that several of the missense and splicing variants classified as pathogenic in those studies were found in association with chain terminating changes in our cohort (for example, R2200C and IVS39-25del72bp in JHU111, N116fsX and JHU104, 3792fsX, respectively) [22,27,44]. R2200C was subsequently found in a small fraction of normal chromosomes and observed in homozygosity (personal communication, JGJ), suggesting that it can occur on a normal haplotype. These reports demonstrate the importance of complete sequencing of both genes in order to identify the *bona fide* gene mutation.

The DNA sequence analysis reported here reveals a remarkable degree of sequence variability particularly in *PKD1* and suggests that a substantial fraction of PKD mutations will be amino acid substitutions. These results also highlight the complexity of assessing the pathogenicity of missense variants particularly in individuals who may harbor multiple such amino acid substitutions. In order to develop the most robust algorithm for classifying missense substitutions, complete sequence data from a reference population of unaffected, ethnically diverse individuals would be invaluable in assessing the pathogenicity of missense changes. These data also would provide an estimate of the frequency of missense changes in normal controls and expand the spectrum of known, non-pathogenic sequence variants. This information would be particularly helpful in analyzing cases where the *a priori* probability of having ADPKD is less than 50%.

In summary, we estimate that in individuals from ADPKD families, approximately 40–60% of DNA tests will yield definitive results. The major limitation of DNA testing in ADPKD is the large number of missense changes that may be challenging to interpret even with the best *in silico* approaches. Since clinical decision-making demands an added level of certainty beyond that required in research situations, Class II results may often be non-diagnostic. Therefore, in some situations, such as the evaluation of prospective renal donors from small ADPKD families, we recommend initial testing of the affected recipient to identify the disease-causing mutation. If a diagnostic mutation is identified, we then proceed with directed testing of the prospective donor. In other situations the possibility of an indeterminate result versus the impact of a positive diagnosis on clinical management must be factored into the decision to pursue DNA testing.

Despite the high frequency of missense mutations, direct DNA testing for ADPKD can provide valuable information in certain clinical circumstances. In the future, sequence analyses of *PKD1* and *PKD2* in normal populations coupled with data from functional research studies should assist in the interpretation of missense variants, thereby expanding the diagnostic utility of molecular genetic testing for ADPKD.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgements

This work was supported by Becas FPI de investigacion (MCYT, Spain) to MGG, R01DK70617 to TW, PKD Center of Excellence DK57325, R37DK48006 to GGG. GGG is the Irving Blum Scholar of the Johns Hopkins University School of Medicine. We thank Ms. Elizabeth Lyden, University of Nebraska Medical Center for assistance with the statistical analysis.

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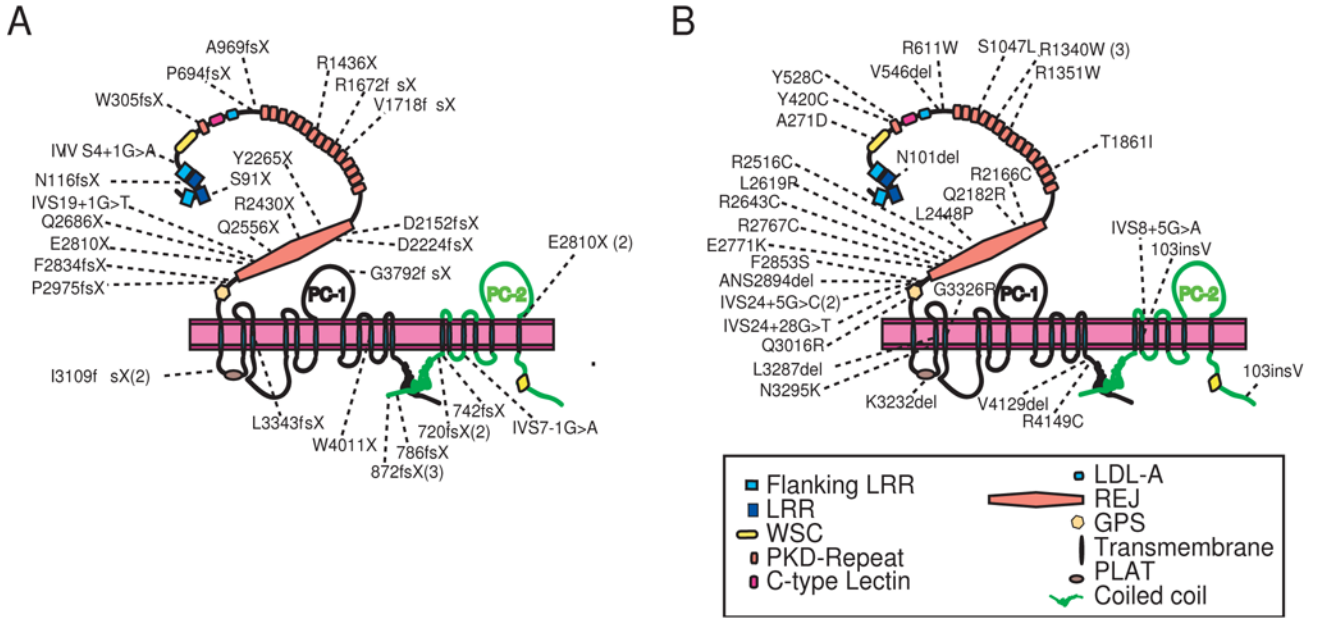
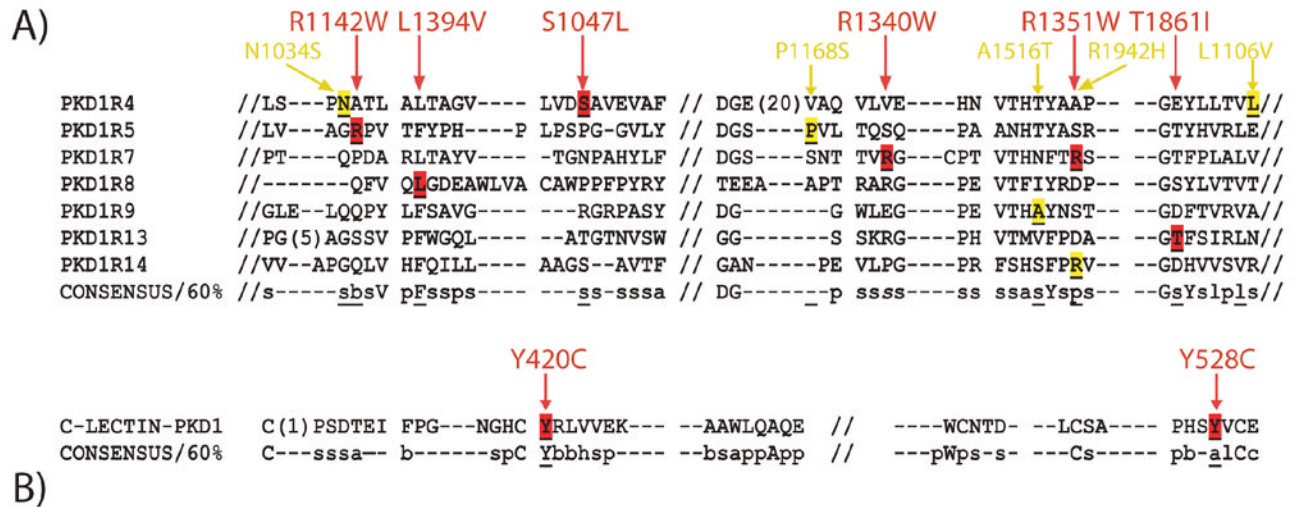


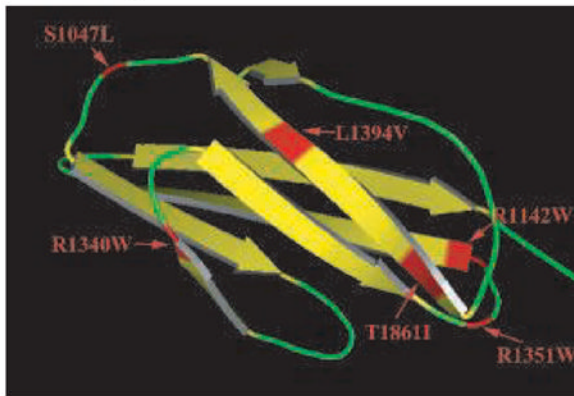
Figure 1. Schematic Representation of Polycystin-1 and 2 (PC-1 and PC-2)

The location of Class I and Class II mutations are indicated in Panels **A** and **B**, respectively. Class I tests contain variants predicted to result in premature truncation of either protein. Class II tests consist of either in-frame deletion/insertions, unique intronic variants predicted to disrupt splicing or at least one amino acid substitution deemed likely to be pathogenic. See text for details.



B)

PKD repeat missense mutations



C-Lectin missense mutations

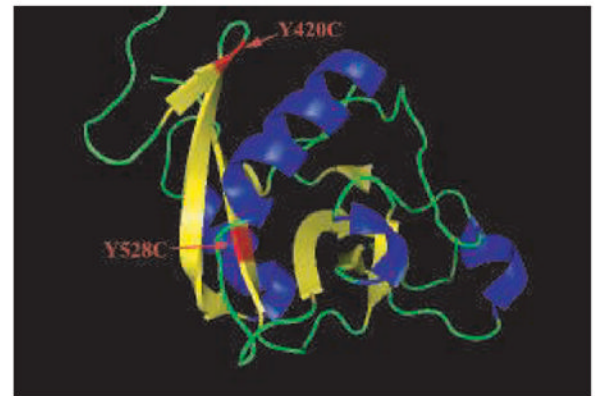


Figure 2. Missense Changes Affecting PKD Repeats and C-Lectin Domain

A) Alignment of the consensus sequences for the PKD repeats and C-Lectin domain. The location of missense changes that disrupt the consensus sequence are shown in red, and those that do not are in yellow. Consensus sequence code: l (Aliphatic), a (aromatic), c (Charged), s (small residue), p (polar residue), b (big residue), h (hydrophobic) and capital letter (corresponding amino acid code). **B)** Ribbon diagrams of PKD repeat and C-Lectin domain. Potentially pathogenic missense changes are demonstrated in red. Helix (blue)-Sheet (yellow)-Loop (green). The secondary structure was determined by PyMol, DeLano Scientific LLC.

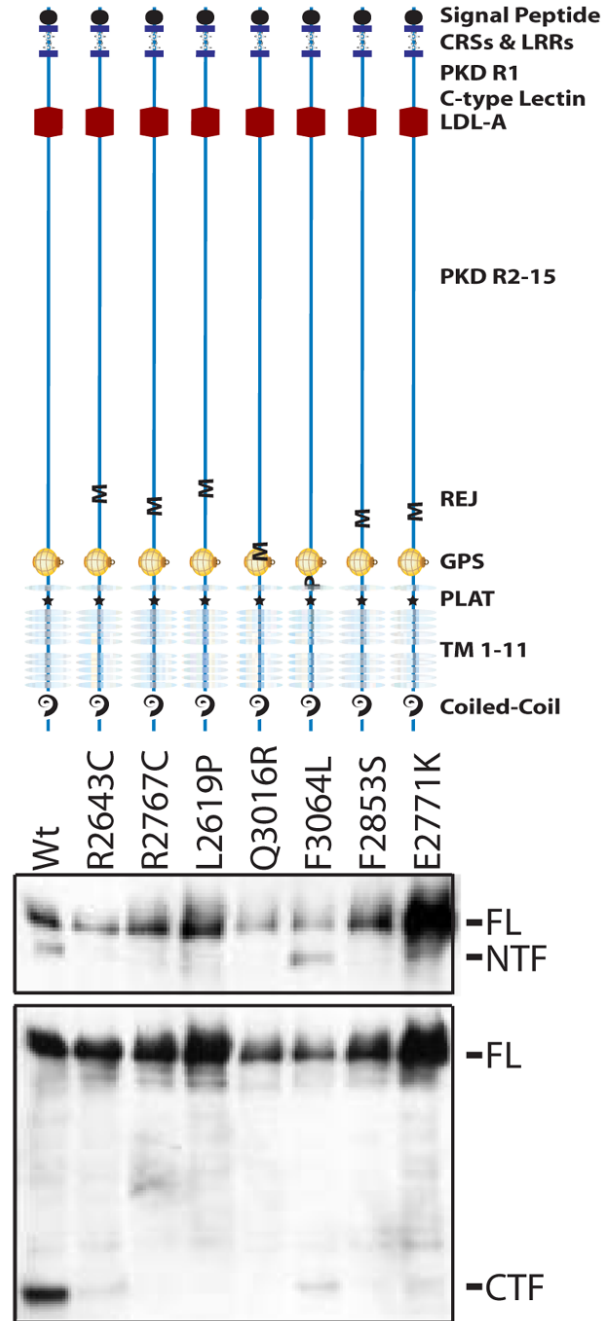


Figure 3. Missense Substitutions Affect PKD1 Cleavage

Top Panel shows a schematic of PKD1 mutant polypeptides with the location of each amino acid substitution indicated by M (missense) or P (polymorphism). HEK cells were transfected with Flag-tagged, full length PKD1 constructs for each mutant protein. Western blots were prepared and loaded in the corresponding lanes below each schematic (middle and bottom Panels). In the middle panel, cell lysates were immunoprecipitated with M2 beads and Western blots were probed with an antibody to the LRR (leucine rich repeat). In the bottom panel, Western blots were probed with an antibody to the C-terminus of PKD1. Absence of the NTF fragment is indicative of aberrant cleavage. Six of the missense changes (E2771K, Q3016R, F2853S, R2643C, R2767C, L2619P) disrupt cleavage. In contrast a PC-1 construct with a

previously described polymorphism (F3064L) cleaved with the wild type pattern. FL: Full-Length, PKD1 NTF: (N-terminal cleavage fragment). Wt: Wild-type PC-1.

Table 1

Study Cohort Characteristics.

% Female ^a	50%
Average Age at time of Test ^a	46.5 (range 1–73y)
% ESRD ^{a,b}	20.7%
Average GFR (ml/min) ^c	68.7 (range 14–126)
% Liver cysts ^a	74.3%
% Vascular complications ^a	9.8%
% Unknown or no Family History ^a	28%

^aData available for N=82 subjects.

^bESRD defined as transplant, dialysis or MDRD (Modification of Diet in Renal Disease) GFR <10.

^cData available for 80 patients.

Table 2

Distribution Of Class I and Class II Tests

	Class I			Class II			Total (%)
	Stop codon	Frameshift	Splice	In frame	Splice	Missense	
<i>PKD1</i>	8 (9.85%)	14 (17.1%)	2 (2.4%)	6 (7.3%)	4 (4.9%)	17 (20.7%)	51 (62.2%)
<i>PKD2</i>	6 (7.3%)	3 (3.65%)	1 (1.2%)	2 (2.4%)	1 (1.2%)	0 (0%)	13 (15.8%)
Total (%)		34 (41.5%)			30 (36.6%)		64 (78%)

Class I tests are those with a definitive pathogenic mutation predicted to result in premature truncation, including stop codons, frameshifts or canonical splice site alterations. Class II tests contained either in-frame deletion/insertions, unique intronic variants predicted to disrupt splicing or at least one amino acid substitution deemed likely to be pathogenic. See text for details.