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ORIGINAL ARTICLE

Comprehensive genetic testing in children with a clinical diagnosis of ARPKD identifies phenocopies

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Abstract

Background Autosomal recessive polycystic kidney disease (ARPKD) is genetically one of the least heterogeneous ciliopathies, resulting primarily from mutations of *PKHD1*. Nevertheless, 13–20% of patients diagnosed with ARPKD are found not to carry *PKHD1* mutations by sequencing. Here, we assess whether *PKHD1* copy number variations or second locus mutations explain these cases.

Methods Thirty-six unrelated patients with the clinical diagnosis of ARPKD were screened for *PKHD1* point mutations and copy number variations. Patients without biallelic mutations were re-evaluated and screened for second locus mutations targeted by the phenotype, followed, if negative, by clinical exome sequencing.

Results Twenty-eight patients (78%) carried *PKHD1* point mutations, three of whom on only one allele. Two of the three patients harbored in trans either a duplication of exons 33–35 or a large deletion involving exons 1–55. All eight patients without *PKHD1* mutations (22%) harbored mutations in other genes (*PKD1* ($n = 2$), *HNF1B* ($n = 3$), *NPHP1*, *TMEM67*, *PKD1*/*TSC2*). Perinatal respiratory failure, a kidney length > +4SD and early-onset hypertension increase the likelihood of *PKHD1*-associated ARPKD. A patient compound heterozygous for a second and a last exon truncating *PKHD1* mutation (p.Gly4013Alafs*25) presented with a moderate phenotype, indicating that fibrocystin is partially functional in the absence of its C-terminal 62 amino acids.

Conclusions We found all ARPKD cases without *PKHD1* point mutations to be phenocopies, and none to be explained by biallelic *PKHD1* copy number variations. Screening for copy number variations is recommended in patients with a heterozygous point mutation.

Keywords Polycystic kidney · CNV · Duplication · Phenocopy · Second locus mutation

Tamás Szabó and Petronella Orosz equally contributed to this work

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Introduction

Autosomal recessive polycystic kidney disease (ARPKD) belongs to the family of primary cilia-related diseases. Though more than 70 genes have been identified in ciliopathies [1], the genetic homogeneity of ARPKD makes it unique: it is principally caused by mutations of *PKHD1* [2]. Recently, mutations of a second gene, *DZIP1L,* have been identified [3] and promoter mutation of *PMM2* was recently described in polycystic kidney disease and hyperinsulinemic hypoglycemia [4]. *PKHD1* is expressed in the renal collecting ducts and in the hepatic bile ducts. The encoded protein, fibrocystin, has a receptor-like structure, and plays a role in maintaining calcium homeostasis [5]. Fibrocystin interacts with polycystin-2, a calcium channel, and is also regulated by polycystin-1 [6–9]. Their role in a common pathway seems to explain the similar renal manifestations of patients with biallelic *PKD1* or *PKD2* mutations [10, 11]. The expression of fibrocystin—besides other ciliary proteins—is regulated by HNF1B. Its haploinsufficiency is a common cause of cystic kidney dysplasia, diagnosed often as a hyperechogenic kidney in utero, which can also mimic perinatally a mild form of ARPKD [12–14]. The clinical presentation of severe ARPKD is highly specific: it is typically diagnosed in utero, with oligohydramnios and extremely enlarged, hyperechogenic kidneys. Thirty percent of the affected children die perinatally because of secondary pulmonary hypoplasia [15]. The diagnosis of mild cases can however be challenging [15]. In these cases, kidneys can be even normal sized, though not smaller than the height-matched median [16]. Hepatic fibrosis (HF) is an obligate feature in ARPKD, secondary to defective remodeling of the ductal plate and hyperplastic biliary ducts [17–19]. Nevertheless, its severity is highly variable, and in most cases, it is difficult to detect by ultrasound scan [18–20]. The association of hepatic fibrosis to cystic kidneys is common in ciliopathies, most typically in *NPHP3*- and *TMEM67*-associated nephronophthisis [21, 22]. Since these can also result in above-average sized hyperechogenic kidneys, their clinical differential diagnosis can also be challenging. Overlapping phenotypes secondary to the influence of second loci or epigenetic factors can further complicate the genetic diagnosis [16, 23, 24].

Genetic confirmation of the diagnosis is demanding for several reasons: *PKHD1* is one of the largest genes consisting of 67 exons in its largest transcript, with more than 700 known mutations (URL: http://www.humgen.rwth-aachen.de) [25]. Furthermore, *PKHD1* copy number variations (CNVs), which are difficult to detect by sequencing, have been described in some cases [15, 25–27]. Finally, the cumulative allele frequency of its mutations is the highest among all ciliopathy genes, resulting in a carrier frequency of 1.5% in the Caucasian population [28]. Therefore, a heterozygous *PKHD1* mutation is a relatively common incidental finding, and does not necessarily mean the trans-association of an undetected mutation in an affected patient.

Despite the principal role of *PKHD1* in ARPKD, no *PKHD1* mutations are identified in 13–20% of the cases by sequencing [15, 16, 27, 29–33]. Here we wanted to find the reason for these cases: whether they result from undetected *PKHD1* mutations or from second locus mutations. We therefore aimed to identify the causal mutations in all cases within a cohort of 36 patients diagnosed with ARPKD by sequencing and multiplex ligation-dependent probe amplification (MLPA) analysis of *PKHD1*, followed, if negative, by mutational screening of second locus mutations and by clinical exome sequencing. We show that the cases negative for *PKHD1* point mutations are phenocopies.

Materials and methods

Patients

Thirty-six unrelated patients from four Hungarian pediatric nephrology centers were included, based on the following criteria: (1) hyperreflective kidneys with microcysts (< 2 cm in diameter) on ultrasound, (2) a kidney length above the 50th percentile (http://radiology-universe.org/calculator/pediatric-kidney-sizes/calculator.php) on at least one side, (3) a transmission compatible with autosomal recessive inheritance, (4) no urinary tract malformation and (5) no extra-renal and -hepatic involvement suggestive of other ciliopathies. None of the families was known to be consanguineous. Parents and patients gave informed written consent.

Screening for *PKHD1* mutations

Genomic DNA was isolated from peripheral blood by standard methods. The exons and intronic junctions were amplified using the primers described by Losekoot et al. [34] and Sanger sequenced on an ABI Prism 310 Genetic Analyzer (Thermo Fischer Scientific, Waltham, MA, USA). Patients without biallelic point mutations were subsequently screened for CNVs by MLPA, performed according to the manufacturer's instructions (MRC-Holland, Amsterdam, the Netherlands). Briefly, DNA was denatured and hybridized overnight with the probe mixes P341 and P342. Hybridized probes were ligated, and amplified with 5′-labeled fluorescent primers. Following separation on an ABI Prism 310 Genetic Analyzer, copy numbers were calculated based on the normalized peak heights following intra- and intersample normalization. Parental samples were screened for the identified mutations to confirm segregation and trans-heterozygosity.

Table 1 Clinical characteristics of patients with PKHD1 mutations. [a]Before end-stage renal disease; [b]Born on 29. gw, [c]born on 31. gw; AS: abnormal structure, CHS: cholestasis, CRF: chronic renal failure, d(s): day(s), dg: diagnosis, Diam.: diameter, ELE: elevated liver enzymes, ESRD: end-stage renal disease, F: fibrosis, Fam: family, gw: gestational week, het: heterozygous, HM: hepatomegaly, hom: homozygous, HSM: hepatosplenomegaly, HT: hypertension; inv.: involvement, IU: intrauterine; KTx: kidney transplant, LC: liver cysts, LTx: liver transplant m: maternal, mth(s): month(s), N: normal, NA: not applicable, ND: no data, p: paternal, P/Pt: patient, Resp: respiratory, RF: renal failure, SDS: standard deviation score, w(s): week(s), yr(s): year(s)

Fam.	Pt	PKHD1 mutation	Exon	Amino acid change	Last follow-up/death (age)	Resp. failure	Age at dg of kidney inv.
F1	P1	c.107C > T hom (m,p)	3	p.Thr36Met	KTx:4 yrs	–	neonate
F2	P2	c.6992 T > A het (p)	43	p.Ile2331Lys	8 yrs	+	neonate
		c.7916C > A het (m)	51	p.Ser2639*			
F3	P3	c.8935C > T het (m)	58	p.Arg2979*	† 6 ds	+	IU
		c.7916C > A het (p)	51	p.Ser2639*			
F4	P4	c.107C > T het(p)	3	p.Thr36Met	8 yrs	+	1 mth
		c.6992 T > A het (m)	43	p.Ile2331Lys			
F5	P5	c.10658 T > C het (p)	65	p.Ile3553Thr	11 yrs	–	4 yrs
		c.7122delT het (m)	46	p.Phe2374Leufs*41			
F6	P6	c.107C > T het (p)	3	p.Thr36Met	† 3 mths	+	neonate
		c.474G > A het (m)	7	p.Trp158*			
F7	P7	c.7916C > A hom (m,p)	51	p.Ser2639*	† 7 ws[b]	+	IU
F8	P8	c.107C > T hom (m,p)	3	p.Thr36Met	† 6 ws	+	neonate
F9	P9	c.107C > T het	3	p.Thr36Met	† 3 ds	+	neonate
		c.7916C > A het	51	p.Ser2639*			
F10	P10	c.5513A > G het (p)	34	p.Tyr1838Cys	KTx:4 yrs	+	neonate
		c.3463C > T het (m)	30	p.Gln1155*			
F11	P11	c.3747 T > G het (p)	32	p.Cys1249Trp	12 yrs	+	IU (32.gw)
		c.5513A > G het (m)	34	p.Tyr1838Cys			
F12	P12	c.7916C > A het (m)	51	p.Ser2639*	KTx:25 yrs	–	7 yrs
		c.10658 T > C het (p)	65	p.Ile3553Thr			
F13	P13	c.547C > T het (p)	8	p.Gln183*	14 yrs	–	IU (30.gw)
		c.4870C > T het (m)	32	p.Arg1624Trp			
F14	P14	c.5_8delCTGC het (p)	2	p.Ala3Glyfs*2	15 yrs	–	5 mths
		c.12036delA het (m)	71	p.Gly4013Alafs*25			
F15	P15	c.107C > T het (p)	3	p.Thr36Met	6 yrs	+	neonate
		c.8552 T > C het (m)	55	p.Ile2851Thr			
F16	P16	c.664A > G het (m)	9	p.Ile222Val	8 yrs	–	infancy
		c.5513A > G het (p)	34	p.Tyr1838Cys			
F17	P17	c.107C > T het (p)	3	p.Thr36Met	5 yrs	–	3 mths
		c.664A > G het (m)	9	p.Ile222Val			
F18	P18	c.2341C > T het (p)	23	p.Arg781*	17 yrs	–	16 yrs
		c.10621A > T het (m)	65	p.Asn3541Tyr			
F19	P19	c.7916C > A hom (m,p)	51	p.Ser2639*	† 3 ws	+	IU
F20	P20	c.4328G > A het (m)	32	p.Cys1443Tyr	4,5 yrs	+	neonate
		c.8870 T > C het (p)	58	p.Ile2957Thr			
F21	P21	c.107C > T het	3	p.Thr36Met	† 1 d	+	IU (32.gw)
		c.5088delTG het	32	p.Gly1696fs*1			
F22	P22	c.107C > T hom (m,p)	3	p.Thr36Met	KTx:1.8 yrs	+	IU (33.gw)
F23	P23	c.107C > T hom (m,p)	3	p.Thr36Met	† 6 ws	+	neonate
F24	P24	c.107C > T het (m)	3	p.Thr36Met	3 yrs	+	5 mths
		c.6992 T > A het (p)	43	p.Ile2331Lys			
F25	P25	c.2167C > T het	22	p.Arg723Cys	6 yrs	+	neonate
		c.8870 T > C het	58	p.Ile2957Thr			
F26	P26	c.9370C > T het (m)	59	p.His3124Tyr	KTx:11 yrs	+	3 mths
		c.(5236 + 1_5237–1)_(5751 + 1_5752–1)dup, het	33–35	abnormal transcript			
F27	P27	c.1-43098_8643-31del het (p)	1–55	no transcript	† 10 ws[c]	+	IU
		c.10174C > T het (m)	61	p.Gln3392*			
F28	P28	c.8114delG het (m)	5	p.Gly2705Valfs*11	26 yrs	–	4 yrs

Fam.	Kidney length at dg [SDS, right-left] (age)	Diameter of the largest renal cyst (age)	Last kidney length[a] [SDS, right-left] (age)	Renal function (age)	HT (age)	Liver involvement (age)
F1	+6.55;+8.67 (6 mths)	10 mm (6 mths)	ND	ESRD (2 yrs)	+ (6 mths)	HM, ELE (0.5 yr)
F2	+8.45;+8.45 (1 d)	6 mm (1 d)	ND	CRF II. (8 yrs)	+ (1 mth)	CHS, HM, ELE, F (2 yrs)
F3	+8.71;+8.71 (5 ds)	18 mm (5 ds)	NA	RF (6 ds)	–	CHS, LC, HM (4ds)
F4	+2.4;+3.07 (2 mths)	<10 mm (2 mths)	+4.08;+5.83 (6 yrs)	N (8 yrs)	+ (1 d)	HM, AS (2 mths)
F5	+3.1;+3.1 (4 yrs)	2 mm (4 yrs)	+2.59;+1.05 (9 yrs)	N (11 yrs)	–	LC (4 yrs)

Table 1 (continued)

F6	+12.82;+12.82 (4 ds)	3 mm (4 ds)	+8.96;+8.96 (3 mths)	ESRD (3 mths)	+ (1 d)	mild HM (4 ds)
F7	+2.42;+2.42 (4 ds)	ND	ND	RF (4 ws)	–	HM (4 ds)
F8	+14.42;+14.42 (4 ds)	5 mm (1 mth)	ND	RF (6 ws)	+ (1 d)	–
F9	+6.47;+5.09 (1 d)	4 mm (1 d)	NA	NA	–	mild HM, ELE, AS (1 d)
F10	+12.36;+12.36 (1 d)	4 mm (3 ds)	+7.88;+7.88 (3 yrs)	ESRD (4 yrs)	+ (1 w)	LTx:4 yrs
F11	+9;+9 (1 mth)	6 mm (1 mth)	+8.77;+6.59 (12 yrs)	CRF IV. (12 yrs)	+ (3 mths)	ELE (1 mth)
F12	+4.85;+4.85 (10 yrs)	5 mm (10 yrs)	+4.46;+4.46 (18.5 yrs)	ESRD (25 yrs)	+ (10 yrs)	LTx:25 yrs
F13	+0.05;+1.8 (3 mths)	6 mm (8 yrs)	+5.15;+7.5 (14 yrs)	N (14 yrs)	–	–
F14	+2.59;+3.8 (5 mths)	1 mm (9 yrs)	+8.28;+10.08 (15 yrs)	CRF II. (15 yrs)	+ (3 yrs)	–
F15	+9.69;+9.69 (5 mths)	9 mm (5 mths)	+19.16;+19.16 (6 yrs)	CRF III. (6 yrs)	+ (1 d)	LC (1 d), AS, HM (3 yrs)
F16	+5.24;+5.24 (3 yrs)	4 mm (3 yrs)	+3.83;3.83 (8 yrs)	N (8 yrs)	–	–
F17	+3.8;+3.8 (4 mths)	10 mm (4 mths)	+6.21;+6.21 (5 yrs)	N (5 yrs)	–	–
F18	+1.59;+3.64 (16 yrs)	11 mm (16 yrs)	ND	CRF II. (17 yrs)	–	AS (16 yrs)
F19	+12.36;+12.36 (1 d)	8 mm (1 d)	+16.79;+16.79 (1 w)	RF (1 w)	+ (1 d)	–
F20	+4.01;+4.01 (4 mths)	3 mm (4 mths)	+10.31;9.1(4.5 yrs)	CRF II. (4.5 yrs)	+ (1d)	AS (4 mths), F (10 mths), HSM (1 yr)
F21	+18.87;+18.87 (1 d)	4 mm (1 d)	NA	NA	ND	–
F22	+4.79;+4.79 (8 ds)	1 mm (8 ds)	+21.95;+21.95 (1 yr)	ESRD (1 yr)	+ (1 d)	F (1 d), ELE (3 mths)
F23	+15.09;+15.09 (2 ds)	4 mm (2 ds)	ND	RF (6 ws)	+ (1 d)	–
F24	+0.2;+0.2 (6 mths)	4 mm (6 mths)	−1.75;-1.75 (3 yrs)	CRF II (1.5 yrs)	–	–
F25	+5.92;+5.92 (5 mths)	8 mm (5 mths)	+10.6;+10.6 (6 yrs)	ESRD (6 yrs)	+ (1 yr)	HSM (5 mths), LC (3 yrs)
F26	+9.24;+4.48 (3mths)	10 mm (3 mths)	ND	ESRD (11 yrs)	+ (3 mths)	LC, HM (3 mths)
F27	+4,1;+4,1 (1 d)	ND	ND	RF (10 ws)	+ (1 d)	–
F28	+7.26;+3.26 (4 yrs)	10 mm (4 yrs)	+3.73;+3.73 (26 yrs)	CRF II. (26 yrs)	+ (17 yrs)	F, HM, ELE (4 yrs)

Screening for second locus CNVs

Patients without biallelic *PKHD1* mutations were re-evaluated based on their most recent phenotype, and were screened for second locus mutations accordingly. Deletions of *NPHP1* and *HNF1B* were tested by QMPSF analysis as described previously [12, 35]. Similarly, continuous gene deletion of *TSC2* and *PKD1* was screened by QMPSF analysis, with the primers listed in Suppl. Table 1, according to the protocol of *NPHP1*-QMPSF [35] with the differences detailed in Suppl. Methods. The deletion of *PKD1* was validated with MLPA, using the SALSA MLPA probemix P352 PKD1-PKD2, performed according to the manufacturer's instructions (MRC-Holland, Amsterdam, the Netherlands).

Screening for second locus (*PKD1*, *PKD2*, *HNF1B* and *TMEM67*) small-scale mutations

Coding exons and intronic junctions of *HNF1B* and *TMEM67* were Sanger sequenced. A dominant polycystic kidney panel was designed using the Ion AmpliSeq Designer version 4.2.1 (Thermo Fischer Scientific, Waltham, MA). Sample enrichment was performed by the Ion AmpliSeq Library Kit (Thermo Fischer Scientific). Samples were barcoded and sequenced on IonTorrent 316 chip (Thermo Fischer Scientific). More than 95% of the target sequence was covered at least 50-fold. Putative disease-causing genetic variants were validated by Sanger sequencing.

Clinical exome sequencing

Clinical exome sequencing was performed as described previously [36]. More than 95% of the target sequence was covered at least 20-fold. Reads were mapped against the human NCBI37/hg19 reference genome. DNA alignment and sequence variant analysis were carried out using NextGene Software version 2.4.2 (SoftGenetics, State College, PA). Data from the proband was filtered for coding and splicing region mutations of known/putative genes associated with cystic kidney diseases.

Results

PKHD1 mutations

Of the 36 unrelated patients with a clinical diagnosis of ARPKD, 27 (75%) were found to carry biallelic *PKHD1* mutations (Table 1). Among them, 25 patients carried biallelic point mutations (P1–25) and two were compound heterozygous for a point mutation and either a duplication of exons 33–35 (P26) or a large deletion encompassing exons 1 to 55 (P27). Furthermore, one patient (P28) was found to carry a single heterozygous frameshift mutation. No *PKHD1* mutation was found in 8 families (Table 2, Fig. 1).

Two mutations, p.Thr36Met and p.Ser2639* were found frequently, in 15/54 (28%) and in 8/54 (15%) of the mutated alleles, respectively. Seven mutations were novel: besides the duplication of exons 33–35 and the deletion of exons 1–55, three truncating mutations (c.5_8delCTGC, p.Ala3Glyfs*2;

Table 2 Clinical characteristics of patients with second locus mutations. [a]Before end-stage renal disease; [b]Patient had GBS perinatal sepsis; [c]P36 and P37 are a sibling pair and for the calculations, genotype information from only one of them was used. d: day, dg: diagnosis, CRF: chronic renal failure, ELE: elevated liver enzymes, ESRD: end-stage renal disease, F: fibrosis, Fam: family, gw: gestational week, het: heterozygous, hom: homozygous, HT: hypertension, inv.: involvement, IU: intrauterine, KTx: kidney transplant, m: maternal, mth(s): month(s), N: normal, NA: not applicable, p: paternal, P/Pt: patient, Resp: respiratory, SDS: standard deviation score, w(s): week(s), yr(s): year(s)

Fam.	Pt	Second locus mutation	Exon	Amino acid change	Last follow-up (age)	Resp. failure	Age at dg of kidney inv.	Kidney length at dg [SDS, right-left] (age)	Diameter of the largest renal cyst (age)	Last kidney length [a] [SDS, right-left] (age)	Renal function (age)	HT (age)	Liver involvement (age)
F29	P29	PKD1 c.12310_12313delGTTA het (de novo)	5	p.Val4104Phefs*93	4 yrs	–	IU	+3.26;+3.26 (7 mths)	3 mm (7 mths)	+2.17;+3.23 (4 yrs)	N (4 yrs)	–	–
F30	P30	PKD1 c.920delT het (de novo)	45	p.Phe307Serfs*27	3.5 yrs	–	IU (32.gw)	+2.19;+0.24 (1 d)	15 mm (1 d)	+6.93;+0.95 (3.5 yrs)	N (3.5 yrs)	–	–
F31	P31	PKD1/TSC2 16:g.(?_2088232)_(2134081_?)del het. (de novo)	TSC2: 41–42, PKD1: 1–46	–	6.5 yrs	–	6 ws	+0.44;+0.54 (3.5 yrs)	10 mm (2 mths)	+0.42;+0.42 (6.5 yrs)	N (6.5 yrs)	–	–
F32	P32	HNF1B(?_-1)_(*1_?)del het (de novo)	1–9	–	9 yrs	–	IU (16.gw)	+1.32;+1.32 (2 mths)	8 mm (2 mths)	+2.82;+2.82 (9 yrs)	N (9 yrs)	–	–
F33	P33	HNF1B(?_-1)_(*1_?)del het (de novo)	1–9	–	6 yrs	–	neonate	+1.62;+1.62 (1 d)	9 mm (5 yrs)	+0.15;+0.5 (6 yrs)	N (6 yrs)	–	–
F34	P34	HNF1B(?_-1)_(*1_?)del het (de novo)	1–9	–	11 yrs	–	IU	−0.1;+0.23 (3 mths)	3 mm (3 mths)	+0.53;+0.53 (10 yrs)	CRF II. (10 yrs)	–	ELE (7 yrs)
F35	P35	NPHP1 (?_-1)_(*1_?)del het (m)	1–20	–	KTx: 12.5 yrs	–	11.5 yrs	+0.42;+0.42 (11.5 yrs)	10 mm (11.5 yrs)	NA	ESRD (11.5 yrs)	+ (11.5 yrs)	ELE (11.5 yrs)
		NPHP1 c.(1810 + 1_1811–1)_(*1_?)del (p)	18–20-	–									
F36	P36 [c]	TMEM67 c.1843 T > C hom	18	p.Cys615Arg	KTx: 9 yrs	+ [b]	IU	+1.29;+1.8 (1 mth)	9 mm (1 mth)	−4.74;-4.74 (9 yrs)	ESRD (9 yrs)	–	–
F36	P37 [c]				20 yrs	–	3 mths	+1.51;+1.51 (3 mths)	2 mm (9 yrs)	−2.88;-2.78 (18 yrs)	ESRD (19 yrs)	–	ELE (11 yrs), F (20 yrs)

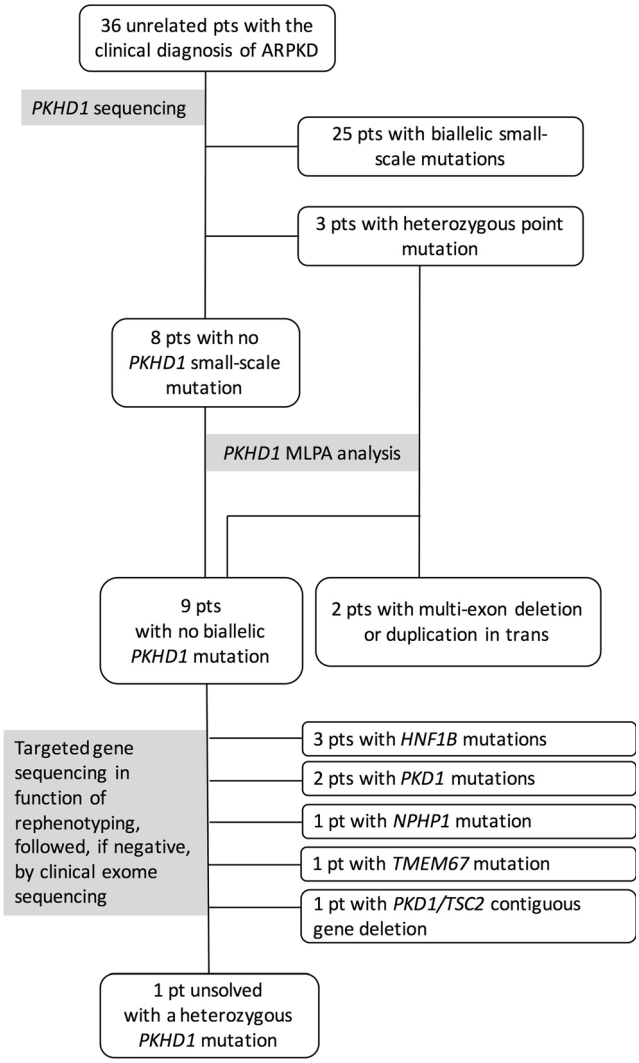

Fig. 1 Mutational screening procedure in children with the clinical diagnosis of autosomal recessive polycystic kidney disease (ARPKD)

c.5088delTG, p.Gly1696fs*1; c.12036delA, p.Gly4013Alafs*24) and two missense mutations (c.4328G > A, p.Cys1443Tyr and c.10621A > T, p.Asn3541Tyr). We considered these two latter to also be pathogenic, because neither is reported in the gnomAD database, both affect amino acids conserved in mammals and are predicted to be pathogenic by Polyphen-2 (score: 1.0 and 0.981, respectively) and MutationTaster.

Second locus mutations

All eight families without *PKHD1* mutations were found to carry second locus mutations (Table 2, Fig. 1). Three children diagnosed with hyperechogenic, normal-sized kidneys in utero or in infancy carried a de novo deletion of *HNF1B*. The renal morphology of two children became suggestive of ADPKD between 2 and 4 years of age. They both harbored de novo *PKD1* mutations. One patient (P31) was diagnosed with tuberous sclerosis at the age of 4 years, and carried a de novo *TSC2*/*PKD1* deletion. Finally, the phenotype of a patient (P35) diagnosed at the age of 11 years with end-stage renal disease was suggestive of juvenile nephronophthisis. He was compound heterozygous for a complete and a partial deletion of *NPHP1*, as described recently [35]. A sibling pair (P36, P37), diagnosed with hyperechogenic kidneys and hepatic fibrosis, was homozygous for the frequent *TMEM67* missense mutation, p.Cys615Arg [21] (for the calculations, genotype information from only one of them was used). They had no neurological involvement. The patient with a heterozygous *PKHD1* mutation (P28) was not found to carry a second locus mutation, even by clinical exome sequencing, which also well covered the coding regions of *NPHP2, NPHP3,* and *WDR19*/*NPHP13*. The mutational screening procedure is summarized in Fig. 1.

Phenotype of patients with *PKHD1* and second locus mutations

Of the 27 patients with *PKHD1* mutations, 19 children (70%) developed perinatal respiratory failure, and nine (33%) died perinatally (< 3 months of age) (Table 1). In contrast, only one of the nine patients (from eight families) with second locus mutations had a transient perinatal respiratory failure, secondary to an infection (Table 2). Among the perinatal cases who survived beyond that period, all but one of the ten children with *PKHD1* mutations developed hypertension by the age of 1 year, compared with none of the six patients with second locus mutations (Table 1). A mean kidney length above + 4 SD at diagnosis was also specific for *PKHD1*-associated ARPKD: 19 of 27 patients (70%) with biallelic *PKHD1* mutations, but none of the nine patients with second locus mutations had such an enlarged kidney. Based on these data, the phenotype of the patient with a single heterozygous *PKHD1* mutation (P28) is highly suggestive of a moderate *PKHD1*-associated ARPKD: she developed hypertension at an early stage, as well as highly enlarged kidneys and hepatic fibrosis. There was no difference in the renal survival between patients with *PKHD1* mutations who survived the perinatal period and the heterogeneous group of patients with second locus mutations (Tables 1 and 2, Fig. 2).

Discussion

Here we aimed to investigate the reason for the high proportion of ARPKD cases negative for *PKHD1* point mutations. Our *PKHD1*-positive rate of 78%—including a patient with a single heterozygous mutation—corresponds well to previous reports [15, 16, 27, 29, 32, 33]. We first assessed the potential role of *PKHD1* CNVs, but found no biallelic CNVs in the *PKHD1* negative cases. This finding was in accordance with

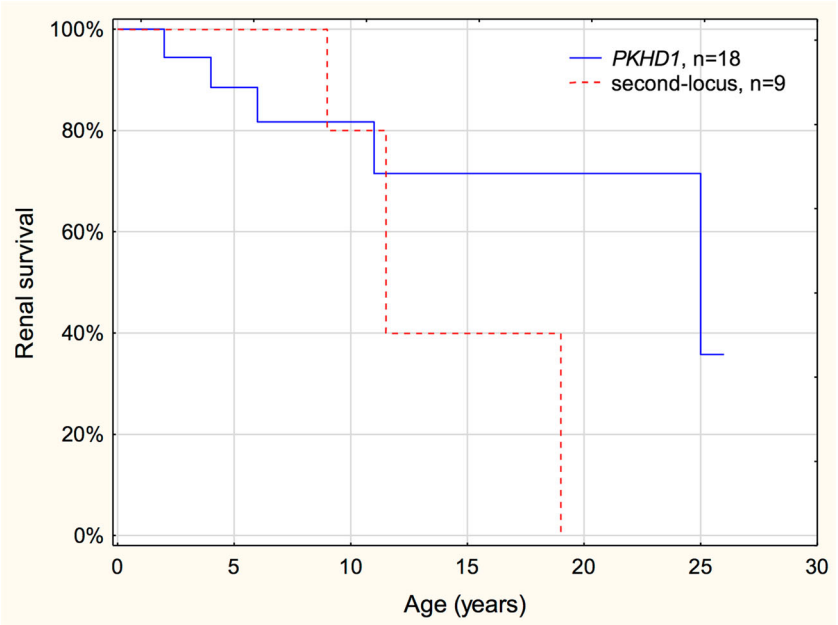

Fig. 2 Renal survival of patients with *PKHD1* and second locus mutations. No difference was found in the renal progression between patients with *PKHD1* and second locus mutations ($p = 0.51$). Among patients with second locus mutations, those with *PKD1* and *HNF1B* mutations were young (≤ 11 years), and patients with nephronophthisis reached end-stage renal disease between 9 and 19 years of age, giving a worse renal survival curve than generally expectable

the low prevalence of *PKHD1* CNVs in other cohorts [15, 16, 26, 27, 30, 31]. We only found a three-exon duplication and a large deletion in two out of three patients with a single heterozygous *PKHD1* point mutation. Duplications are extremely rare; here, we present only the second such case [26]. These results emphasize that screening for CNVs is primarily important in patients with a heterozygous *PKHD1* point mutation.

To identify all mutations of the coding regions, we used the most sensitive but also labor- and time-consuming methods of Sanger sequencing and MLPA analysis. However, excluding the causal role of *PKHD1* intronic and promoter mutations or rearrangements in patients with or without a single heterozygous mutation is especially challenging by direct genetic tests. Therefore, we aimed to identify the causal mutations in all patients with a clinical diagnosis of ARPKD, and thus exclude the causal role of *PKHD1* indirectly. We found all patients without *PKHD1* mutations to carry causal mutations in second loci, by re-evaluation of the phenotype and targeted mutation screening, indicating that 22% of the initial clinical ARPKD diagnoses were false. We failed to identify in the third child with a heterozygous *PKHD1* mutation a second locus mutation, even following clinical exome sequencing that also covered the promoter region of *PMM2* [4]. This is in accordance with the phenotype which is strongly suggestive of *PKHD1*-associated ARPKD. Her case thus points on the difficulty in identifying some *PKHD1* mutations even by the combined approach of sequencing and MLPA, and suggests a potential role of an intronic or a regulatory *PKHD1* mutation.

The successful identification of second locus mutations in negative cases by direct genetic tests emphasizes the importance of their thorough re-phenotyping. The differential diagnosis of mild and moderate forms without extremely enlarged kidneys and respiratory failure can be challenging at diagnosis. Within this cohort, an infant-onset hypertension was highly suggestive of *PKHD1*-associated ARPKD.

In accordance with the literature, the phenotype of patients with *PKHD1* mutations strongly correlated with the causal mutations; no patient survived the perinatal period with biallelic loss-of-function mutations [16, 37, 38]. Interestingly, a patient, compound heterozygous for a second and a last exon truncating mutation, p.Gly4013Alafs*24 (P14), presented with a moderate phenotype: she was diagnosed at the age of 5 months and had a normal GFR at the age of 9 years, indicating that the loss of the C-terminal 62 amino acids of fibrocystin does not cause complete loss of function. The intracellular C-terminal part of fibrocystin consists of 192 amino acids and is known to modulate the mTOR pathway [39]. It also contains the ciliary targeting sequence (p.3876_3893CLVCCWLKRSKSRKTKPE), that remains unaffected in the Gly4013Alafs*24 fibrocystin [40]. Along the same lines as the hypomorphic nature of this C-terminal truncation, mice lacking the last exon (exon 67), which encodes the nuclear localization signal and the polycystin 2 binding domain, develop a normal phenotype [41].

In conclusion, we found one quarter of the ARPKD cases to be phenocopies, caused by second locus mutations. Our data suggest that perinatal respiratory failure, a kidney length > + 4 SD and early-onset hypertension increase the likelihood of *PKHD1*-associated ARPKD. The phenotype of cases that are negative on *PKHD1* sequencing should first be re-evaluated. We recommend screening for *PKHD1* CNVs in patients with a heterozygous point mutation and in families with an unequivocal phenotype.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Ethical approval All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards. Parents and patients gave informed written consent.

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