http://www.kidney-international.org

Genotype–phenotype correlations in fetuses and neonates with autosomal recessive polycystic kidney disease

Erick Denamur[1,9], Anne-Lise Delezoide[2], Corinne Alberti[3], Agnès Bourillon[4], Marie-Claire Gubler[5,9], Raymonde Bouvier[6], Olivier Pascaud[1], Jacques Elion[1], Bernard Grandchamp[4], Laurence Michel-Calemard[7], Pascale Missy[3], Isabelle Zaccaria[3], Hervé Le Nagard[3], Bénédicte Gerard[1,4,9], Chantal Loirat[8,9] and the Société Française de Foetopathologie[10]

[1]Assistance Publique-Hôpitaux de Paris, Université Paris Diderot, Hôpital Robert Debré, Laboratoire de Biochimie Génétique, INSERM U722, Paris, France; [2]Assistance Publique-Hôpitaux de Paris, Université Paris Diderot, Hôpital Robert Debré, Laboratoire de Biologie du Développement, Paris, France; [3]Assistance Publique-Hôpitaux de Paris, Université Paris Diderot, Hôpital Robert Debré, Unité d'Epidémiologie Clinique, INSERM CIE 5, Paris, France; [4]Assistance Publique-Hôpitaux de Paris, Université Paris Diderot, Hôpital Bichat, Laboratoire de Biochimie Hormonale et Génétique, Paris, France; [5]INSERM U574, Université Paris Descartes, Hôpital Necker, Paris, France; [6]Hospices Civils de Lyon, Université Claude Bernard, Laboratoire d'Anatomie Pathologique, Bron Cedex, France; [7]Hospices Civils de Lyon, Université Claude Bernard, Endocrinologie Moléculaire et Maladies Rares, Bron Cedex, France; [8]Assistance Publique-Hôpitaux de Paris, Université Paris Diderot, Hôpital Robert Debré, Service de Néphrologie, Paris, France and [9]Centre de Référence MARHEA, Maladies Rénales Héréditaires de l'Enfant et de l'Adulte, France

The prognosis of autosomal recessive polycystic kidney disease is known to correlate with genotype. The presence of two truncating mutations in the *PKHD1* gene encoding the fibrocystin protein is associated with neonatal death while patients who survive have at least one missense mutation. To determine relationships between genotype and renal and hepatic abnormalities we correlated the severity of renal and hepatic histological lesions to the type of *PKHD1* mutations in 54 fetuses (medical pregnancy termination) and 20 neonates who died shortly after birth. Within this cohort, 55.5% of the mutations truncated fibrocystin. The severity of cortical collecting duct dilatations, cortical tubule and glomerular lesions, and renal cortical and hepatic portal fibrosis increased with gestational age. Severe genotypes, defined by two truncating mutations, were more frequent in patients of less than 30 weeks gestation compared to older fetuses and neonates. When adjusted to gestational age, the extension of collecting duct dilatation into the cortex and cortical tubule lesions, but not portal fibrosis, was more prevalent in patients with severe than in those with a non-severe genotype. Our results show the presence of two truncating mutations of the *PKHD1* gene is associated with the most severe renal forms of prenatally detected autosomal recessive polycystic kidney disease. Their absence, however, does not guarantee survival to the neonatal period.

Kidney International (2010) **77,** 350–358; doi:10.1038/ki.2009.440; published online 25 November 2009

KEYWORDS: autosomal recessive polycystic kidney disease; congenital hepatic fibrosis; foetopathology; *PKHD1*; prenatal diagnosis

Correspondence: *Chantal Loirat, Service de Néphrologie, Hôpital Robert Debré, 48 bd Sérurier, 75 019 Paris, France.*
E-mail: chantal.loirat@rdb.aphp.fr

[10]The members of the Société Française de Foetopathologie who provided the renal and hepatic tissue specimens are: J. Barbet, Hôpital Saint Vincent de Paul, Paris; A.M. Beaufrère, CHU Clermont Ferrand; C. Berchel, CHU Angers; B. Bessières, Institut de Puériculture Paris; S. Boudjemaa, Hôpital Trousseau, Paris; A. Buenerd, CHU Lyon; D. Carles, CHU Pellegrin, Bordeaux; A. Clemenson, CHU St Etienne; P. Dechelotte, CHU Clermont-Ferrand; L. Devisme, CHU Lille; F. Dijoud, CHU Lyon; O. Espérandieu, CH Orléans; C. Fallet, Hôpital Ste Anne, Paris; M. Gonzalès, Hôpital Saint Antoine, Paris; Y. Hillion, CH Poissy; B. Jacob, CHU Caen; M. Joubert, CHU Nantes; P. Kermanach, CH Annecy; A. Lallemand, CHU Nice; A. Laquerrière, CHU Rouen; N. Laurent, CHU Dijon; A. Liprandi, CHU Marseille; L. Loeuillet, CHU Rennes; P. Loget, CHU Le Mans; J. Martinovic, Hôpital Necker, Paris; F. Ménez, Hôpital Robert Debré, Paris; F. Narcy, Hôpital Cochin, Paris; J.J. Roux, CH Chambéry; C. Rouleau-Dubois, CHU Montpellier; M. Sinico, Hôpital Henri Mondor, Paris; J. Tantau, Hôpital Cochin, Paris; A.R. Wann, Hôpital Saint Vincent de Paul, Paris.

Received 11 December 2008; revised 20 September 2009; accepted 29 September 2009; published online 25 November 2009

Autosomal recessive polycystic kidney disease (ARPKD) is characterized by the association of bilateral renal cystic disease and congenital hepatic fibrosis (CHF). ARPKD can involve a wide spectrum of clinical phenotypes, correlated in part with the age at presentation.[1,2] The most severe forms of the disease, accounting for about 40% of cases, are detected early during gestation by ultrasonography that shows tremendously enlarged echogenic kidneys and oligohydramnios. If medical pregnancy termination is not performed, most of these infants die in the perinatal period from respiratory insufficiency due to pulmonary hypoplasia. But

over half of the patients are detected late during pregnancy or are undetected prenataly and survive the perinatal period. More than half of them require kidney transplantation before reaching 20 years of age, whereas others have preserved renal function until adulthood.[1–5] CHF complications such as bleeding esophageal varices and cholangitis, may occur as soon as early childhood or during adulthood, sometimes years after renal transplantation.[6]

ARPKD is caused by mutations in the *PKHD1* gene that encodes the protein fibrocystin expressed in the primary cilia and the basal body of renal and bile ducts epithelial cells.[7–14] Presently, 303 mutations of *PKHD1* have been reported in ARPKD patients (http://www.humgen.rwth-aachen.de/). Several studies have shown that truncating mutations were present in about 55% of the most severe cases—medical pregnancy termination or early demise patients—but only in about 20% of live patients.[10,15–22] But no study ever analyzed the relationship between the severity of histological renal and hepatic lesions and the genotype.

The aim of our study was to investigate the genotype–phenotype correlations in a cohort of 74 fetuses or early demise patients with ARPKD, by analyzing the relations between the histological severity of renal and hepatic lesions and the type of *PKHD1* mutations.

RESULTS

Renal and hepatic histology

According to inclusion criteria, dilatation of medullar collecting ducts, radially oriented, was present in the 73 available kidney specimens. Portal fibrosis and increased number and dilatation of bile ducts were present in the 61 available liver specimens.

The severity of renal lesions was evaluated according to five criteria: (i) The extension in the cortex of the dilatation of collecting ducts, which was graded as either absent, or partial if present only in the inner part of the cortex, with preservation of the nephronic zone and the superficial rows of developing nephrons, or global if affecting the whole cortex up to the subcapsular areas (Figure 1). (ii) The presence of degenerative changes of cortical tubules (proximal and distal convoluted tubules) that may be dedifferentiated, atrophic or reduced in number. According to their extension and severity, lesions were classified as grade 1: absent of significant changes or mild tubular lesions mostly seen in the deep cortex; grade 2: moderate but diffuse tubular lesions; grade 3: massive reduction in the number of nephrons with complete disappearance of proximal tubules (Figure 2a to f). (iii) The absence or presence of proximal tubule dilatation with preservation of a normal epithelium (Figure 2g). (iv) The appearance of the glomeruli: they may be normal or present unspecific changes within interstitial fibrotic areas, from retraction to collapse, often surrounded by a thickened Bowman's capsule. The lesions were classified as absent, partial or global (Figure 2a to h). (v) The change in the cortical interstitium: fibrosis or fibro-oedema was either absent, focal, or diffuse (Figure 2i).

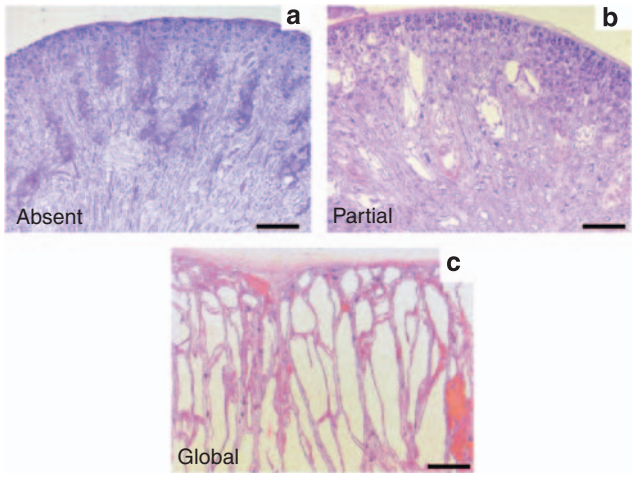

Figure 1 | Categorization of the extension in the renal cortex of the collecting duct dilatation. (**a**) Absent (patient 48); (**b**) partial (patient 6); (**c**) global (patient 49). Histological sagittal sections of fetal kidneys. Hematoxylin and eosin safran staining. Bar = 1 mm. For information on patients, see Supplementary Table S1.

In the liver, the severity of lesions was categorized according to the importance of portal fibrosis, classified as grade 1: presence of mild fibrosis, only touching the largest portal tracts; grade 2: fibrosis of all portal tracts, whatever their size; grade 3: massive fibrosis leading to marked enlargement of all portal tracts with fibrous extensions bridging adjacent portal areas (Figure 3).

Extension of collecting duct dilatation in the cortex was absent in three cases (4%), partial in 35 cases (48%) and global in 35 cases (48%). Cortical tubule lesions were grade 1 in 19 cases (26%), grade 2 in 29 cases (40%), and grade 3 in 25 cases (34%). Proximal tubule dilatation was observed in 26 cases (37%). Glomerular lesions were present in 44 cases (62%) and cortical fibrosis in 24 cases (34%) (Table 1 and Supplementary Table S1). Portal fibrosis was grade 1 in 5 cases (8%), grade 2 in 41 cases (67%), and grade 3 in 15 cases (25%) (Table 1 and Supplementary Table S1).

The degree of extension of collecting duct dilatation within the cortex was significantly correlated with the severity of cortical tubule lesions, glomerular lesions and cortical fibrosis. Proximal tubule dilatation was the only lesion that was significantly more frequent when collecting duct dilatation in the cortex was absent or partial compared with global (Table 2). There was also a correlation, close to significance, between the extension of collecting duct dilatation within the cortex and the grade of portal fibrosis (Table 2).

The degree of extension of collecting duct dilatation within the cortex, the severity of cortical tubule and glomerular lesions significantly increased with gestational age (GA). A similar trend was observed between the severity of cortical fibrosis and GA. Proximal tubule dilatation was the only lesion that was significantly more frequent when GA was $\leqslant 30$ weeks compared with > 30 weeks. The grade of portal fibrosis also significantly increased with GA (Table 1).

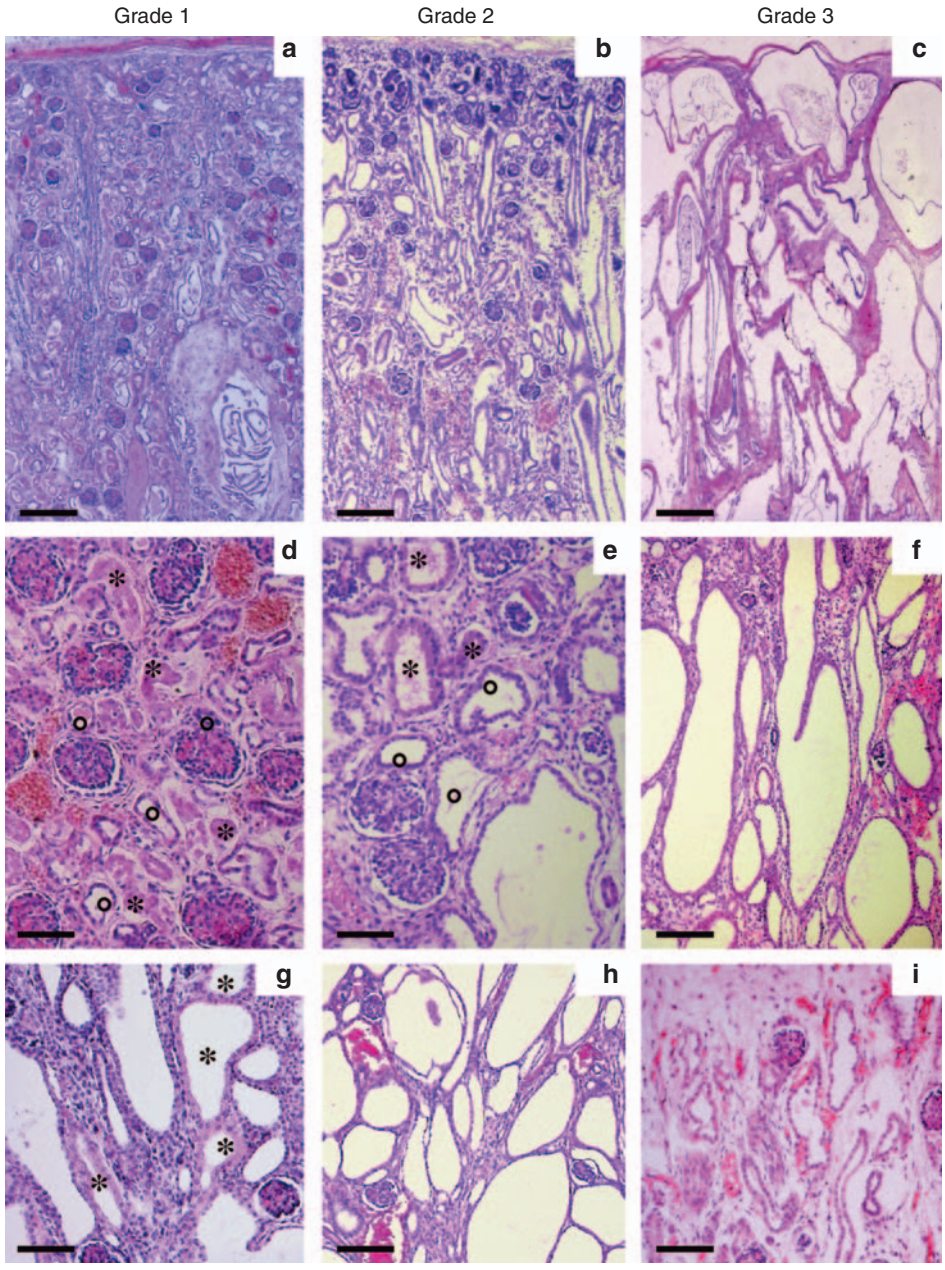

Figure 2 | Categorization of cortical lesions. (**a**–**f**) Grading of cortical tubule lesions with (**a**, **d**): grade 1 (patient 68), despite some degree of autolysis, tubule morphology is preserved; (**b**, **e**): grade 2 (patient 6), epithelial dedifferentiation and atrophy of some cortical tubules leading to irregular dilatation; (**c**, **f**): grade 3 (patients 70 and 12), cortical tubules are scanty and dedifferentiated. (**g**) dilatation of proximal tubules without epithelial lesions (patient 72). (**a**, **b**, **d**–**h**) grading of glomerular lesions with (**a**, **b**, **d**, **e**): normal glomeruli (patients 68 and 6); (**g**) modest retraction of the glomeruli (patient 72); (**f**, **h**) moderate to complete retraction of the glomeruli (patient 12). (**i**) focal cortical interstitial fibrosis (patient 15). Histological sagittal sections of kidneys. Hematoxylin and eosin safran staining. Bar—a, b, c = 300 μm; d, e, f, g, i = 80 μm, h = 150 μm. *Proximal convoluted tubule; °distal convoluted tubule. For information on patients, see Supplementary Table S1.

The impact of GA on histological lesions is illustrated in Figure 4, showing renal and hepatic lesions in two consecutive affected foetuses of the same family (patient 54), at 27 weeks and 13 weeks of GA, respectively.

PKHD1 mutations

A total of 128 mutations were detected, four patients (no. 17, 36, 59, and 60) had three mutations (Supplementary Table S2). The detection rate of the mutations was 83.8% (124 mutated alleles out of 148 expected). Among the 75 distinct identified mutations (Supplementary Table S3), 41 (54.6%) were not previously reported according to the *PKHD1* mutation database (http://www.humgen.rwth-aachen.de/). At least two mutations were detected in 53 patients (71.6%) and one mutation in 18 patients (24.3%). No mutations could be detected in three patients.

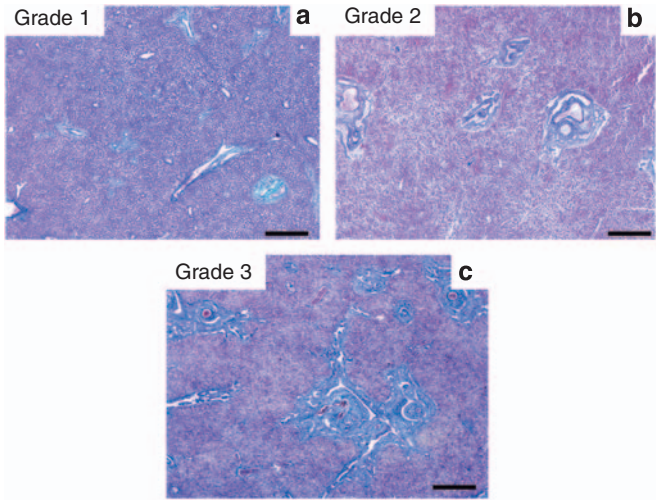

Figure 3 | Categorization of portal fibrosis severity. (**a**) Grade 1 (patient 25); (**b**) grade 2 (patient 6); (**c**) grade 3 (patient 33). Histological sections of livers. Trichrome staining. Bar = 1 mm. For information on patients, see Supplementary Table S1.

Table 1 | Relationships between gestational age and severity of renal and hepatic lesions

	N	GA ⩽30 weeks	GA >30 weeks	*P*-value
Renal lesions				
Collecting duct dilatation in the cortex	73			$<10^{-3}$
Absent		2 (6)	1 (3)	
Partial		26 (72)	9 (24)	
Global		8 (22)	27 (73)	
Cortical tubule lesions	73			0.02
Grade 1		13 (36)	6 (16)	
Grade 2		16 (44)	13 (35)	
Grade 3		7 (19)	18 (49)	
Proximal tubule dilatation	71			$<10^{-3}$
No		16 (44)	29 (83)	
Yes		20 (56)	6 (17)	
Glomerular lesions	71			$<10^{-3}$
Absent		20 (56)	7 (20)	
Partial		11 (31)	9 (26)	
Global		5 (14)	19 (54)	
Cortical fibrosis	71			0.08
Absent		28 (78)	19 (54)	
Focal		6 (17)	9 (26)	
Diffuse		2 (6)	7 (20)	
Portal fibrosis	61			0.002
Grade 1		3 (12)	2 (6)	
Grade 2		22 (84)	19 (54)	
Grade 3		1 (4)	14 (40)	

Abbreviation: GA, gestational age.
Results are expressed as numbers (percentages).
For definition of lesions: see Results, Renal and hepatic histology.

Table 2 | Relationships between the degree of extension of collecting duct dilatation in the cortex and the severity of cortical tubule lesions, proximal tubule dilatation, glomerular lesions, cortical fibrosis and portal fibrosis

Collecting ducts dilatation in the cortex	*N*	Absent	Partial	Global	*P*-value
Cortical tubule lesions	73				$<10^{-3}$
Grade 1		3 (100)	16 (46)	0	
Grade 2		0	18 (51)	11 (31)	
Grade 3		0	1 (3)	24 (69)	
Proximal tubule dilatation	71				$<10^{-3}$
No		0	14 (41)	31 (91)	
Yes		3 (100)	20 (59)	3 (9)	
Glomerular lesions	71				$<10^{-3}$
Absent		3 (100)	23 (68)	1 (3)	
Partial		0	11 (32)	9 (26)	
Global		0	0	24 (71)	
Cortical fibrosis	71				$<10^{-3}$
Absent		3 (100)	30 (88)	14 (41)	
Focal		0	4 (12)	11 (32)	
Diffuse		0	0	9 (26)	
Portal fibrosis	60				0.09
Grade 1		1 (50)	2 (7)	2 (7)	
Grade 2		1 (50)	22 (79)	17 (57)	
Grade 3		0	4 (14)	11 (37)	

Results are expressed as numbers (percentages).
For definition of lesions: see Results, Renal and hepatic histology.

Seventy-one mutations (55.5%) were truncating with 44 frameshifting small deletions/insertions/duplications, 24 nonsense mutations and three splice mutations with exon skipping, leading to out of frame fusion of exons. Fifty-four mutations (42.2%) were missense mutations. One mutation was a missense mutation associated with an in-frame insertion of six bases in exon 16, one mutation was an in-frame deletion of 42 bases in exon 30 and one mutation was a one-base deletion in exon 67 leading to the change of the last 12 amino acids of the fibrocystin, the abolition of the stop codon and the addition of nine amino acids (Supplementary Tables S2 and S3). Parent DNA analysis in the four patients with three mutations showed that the co-inherited mutations were always missense mutations (Supplementary Table S2).

We observed eight patients with homozygous mutations, implicating truncating mutations in all except one (Supplementary Table S2). When parents DNAs were available (five cases), the homozygous character of the mutation was confirmed by testing both parents. In the three remaining cases, intragenic SNPs and intra and flanking extragenic microsatellites showed a homozygous pattern (data not shown), indicating that the mutation was either homozygous, or in combination with a large *PKHD1* deletion.

Mutations were observed all along the gene, scattered in 41 exons. However, three mutations were recurrent: the T36M missense ($n = 13$) in exon 3 and the frameshifts c.5895dupA (L1966fs) ($n = 9$) in exon 36 and c.9689delA (D3230fs) ($n = 13$) in exon 58 (Supplementary Tables S2 and S3). Furthermore, the screening of 6 exons (3, 32, 36, 57, 58, and 61) allowed the identification of 51% of mutations.

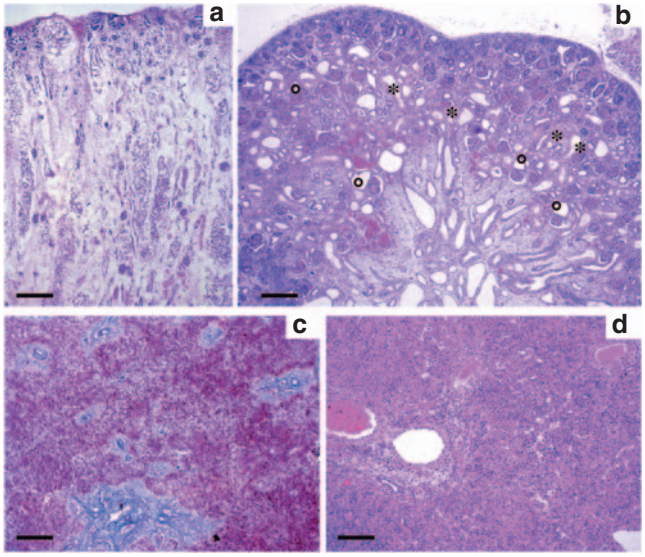

Figure 4 | Correlation of renal and hepatic lesions with gestational age (GA): example from two siblings with different GA. (**a**) Histological sagittal section of the kidney of patient 54 (27 weeks of GA), showing extensive dilatation of collecting ducts, and moderate lesions of cortical tubules (grade 2). (**b**) Histological sagittal section of the kidney of the sibling of patient 54, at 13 weeks of GA; the dilatation of collecting ducts is mainly seen in the medulla; the nephrons are preserved apart from some proximal and distal tubule dilatation. *Proximal convoluted tubule; °distal convoluted tubule. (**c**) Histological aspect of the liver in patient 54, showing numerous dilated bile ducts, enlarged portal tracts and grade 2 portal fibrosis. (**d**) Histological aspect of the liver of sibling at 13 weeks of GA showing very mild dilatation of bile ducts and slight enlargement of portal tract. (**a,b,d**) hematoxylin and eosin safran staining; (**c**) trichrome staining. Bar—a = 300 μm; b, d = 150 μm; c = 1 mm.

Additional 6 exons (9, 14, 18, 19, 22, and 34) led to a detection rate of 64%.

Genotype–phenotype correlation analysis

For this analysis, truncating mutations (T) (nonsense, frameshift and splice mutations) and non-truncating mutations (NT) (missense mutations and diverse in-frame modifications) were considered (Supplementary Table S2). According to the fact that the disease is recessive with a loss of function of the fibrocystin protein, we considered genotype as severe when two truncating mutations were present (T/T, 21 patients) and non-severe if at least one NT mutation was present (41 patients including 20 with NT/T, 12 with NT/NT and 9 with NT/non identified (NI) mutations). Patients with T/NI (nine patients) or NI/NI (three patients) mutations were excluded for the genotype–phenotype correlation analysis as they could have undetected severe mutations such as an intragenic or large *PKHD1* deletion, not screened in our study.

Severe genotype was significantly more frequent in fetuses that underwent medical pregnancy termination at 30 weeks of GA or less (53%) compared to those with GA over 30 weeks (16%, including three medical pregnancy terminations and two post-natal deaths). Conversely, the majority of patients who underwent medical pregnancy termination after 30 weeks of GA or who died after birth had non-severe genotype (Table 3). It is interesting to note that 10 different missense mutations (T36M, I222V, R328G, T1300K, C1692Y, P1791L, R2370G, W2736G, I2957T, and R3240K) were identified among the 16 patients with non-severe genotype who died after birth.

Table 3 | Distribution of gestational age at medical pregnancy termination or death according to genotype (severe or non-severe)

		Genotype		
	N 62	Non-severe *N*=41	Severe *N*=21	*P*-value
Medical pregnancy termination ⩽30 weeks	30	14	16 (53)	0.006
Medical pregnancy termination >30 weeks	14	11	3 (21)	
Post-natal demise	18	16	2 (11)	

Results are expressed as numbers (percentages per row).
For definition of genotypes: see Results, Genotype–phenotype correlation analysis.

When GA was not taken into account, no correlation was found between the severity of genotype and any of the histological criteria of severity (Supplementary Table S4). However, when adjusted to GA, the degree of extension of collecting duct dilatation in the cortex was significantly more important in the patients with severe genotype compared to those with non-severe genotype. The grade of cortical tubule lesions adjusted to GA was also more important in the patients with severe genotype than in those with non-severe genotype (Table 4). Proximal tubule dilatation, glomerular lesions, cortical fibrosis, and hepatic fibrosis adjusted on GA were not significantly different between the two groups of genotype (Table 4).

DISCUSSION

This is the first series of ARPKD patients who analyzed the genotype–phenotype correlations via the relations between the severity of renal and hepatic histological lesions and *PKHD1* mutations.

The severity of the disease in this series of prenatally detected ARPKD was confirmed by the association of severe cortical lesions with the typical dilatation of collecting ducts in the medulla. Extension to the cortex of collecting duct dilatation was observed in 96% of patients and cortical tubule lesions grade 2 or 3 in 74% of the patients. Similarly, portal fibrosis grade 2 or 3 was present in 92% of patients (Table 1 and Supplementary Table S1). It is generally considered that cortical lesions in ARPKD are the consequence of compression by the cystic collecting ducts extending to the cortex. Our series confirms that the severity of cortical tubule lesions, glomerular lesions, and cortical fibrosis were significantly correlated with the degree of extension of

Table 4 | Relationships between genotype (severe or non-severe) and renal lesions and portal fibrosis adjusted to gestational age

		Genotype		
	N	Non-severe	Severe	*P*-value
Renal lesions				
Collecting duct dilatation in the cortex	61			0.01
GA ⩽30 weeks				
Absent or partial	25	14	11 (44)	
Global	5	0	5 (100)	
GA >30 weeks				
Absent or partial	7	7	0	
Global	24	20	4 (17)	
Cortical tubule lesions	61			0.07
GA ⩽30 weeks				
Grade 1	11	4	7 (64)	
Grade 2	15	10	5 (33)	
Grade 3	4	0	4 (100)	
GA >30 weeks				
Grade 1	3	3	0	
Grade 2	11	10	1 (9)	
Grade 3	17	14	3 (18)	
Proximal tubule dilatation	59			0.44
GA ⩽30 weeks				
No	12	5	7 (58)	
Yes	18	9	9 (50)	
GA >30 weeks				
No	25	21	4 (16)	
Yes	4	4	0	
Glomerular lesions	59			0.34
GA ⩽30 weeks				
Absent	17	9	8 (47)	
Partial	11	5	6 (55)	
Global	2	0	2 (100)	
GA >30 weeks				
Absent	3	3	0	
Partial	8	7	1 (13)	
Global	18	15	3 (17)	
Cortical fibrosis	59			0.85
GA ⩽30 weeks				
Absent	24	11	13 (54)	
Focal	5	3	2 (40)	
Diffuse	1	0	1 (100)	
GA >30 weeks				
Absent	13	12	1 (8)	
Focal	9	7	2 (22)	
Diffuse	7	6	1 (14)	
Portal fibrosis	52			0.69
GA ⩽30 weeks				
Grade 1	3	2	1 (33)	
Grade 2	19	10	9 (47)	
Grade 3	1	1	0	
GA >30 weeks				
Grade 1	2	2	0	
Grade 2	15	12	3 (20)	
Grade 3	12	10	2 (17)	

For definition of lesions: see Results, Renal and hepatic histology.
For definition of genotypes: see Results, Genotype–phenotype correlation analysis.

collecting duct dilatation within the cortex (Table 2). It is interesting to note that a correlation close to significance was also found between the degree of portal fibrosis and the extension of collecting ducts in the renal cortex, suggesting that renal and hepatic involvement have a parallel severity in the most severe forms of ARPKD detected prenatally.

A prerequisite before investigating the correlations between histological phenotype and genotype was to analyze the effect of GA on the severity of histological lesions. Our study confirmed that the degree of extension of collecting duct dilatation within the renal cortex, the severity of cortical tubule lesions, glomerular lesions, cortical fibrosis, and portal fibrosis increased significantly with GA (Table 1), a logical issue, but not demonstrated previously in humans, even in the seminal studies of Osathanonah and Potter.[23] The only histological abnormality, which we observed to be more frequent when GA was ⩽30 weeks compared with >30 weeks was the dilatation of proximal tubules (Table 1), which also was more frequent when the extension of collecting duct dilatation within the cortex was absent or partial compared with global (Table 2). Dilatation of proximal tubules has been reported as an early lesion, preceding collecting duct cystic dilatation in human ARPKD fetuses[24] and several mouse models of ARPKD.[25–29] Ward *et al.*[9] have shown that the PKHD1 protein fibrocystin is expressed in embryonic human kidneys not only in the branching ureteric ducts and collecting ducts, but also, with a weaker intensity, in the developing nephrons. In addition, expression of fibrocystin has been shown in human adult[12] and in mouse[30] proximal tubules.

In this series of severe ARPKD, the mutation detection rate was 83.8%, a proportion similar to that reported in series including the most severe forms of the disease.[10,15–18] Two mutations were identified in 71.6% of patients, one in 24.3%, and none in three patients (2.4%). This repartition is similar to that reported by Bergmann *et al.*[16] in families requesting prenatal diagnosis after having lost a child with severe ARPKD. Among the 75 different mutations, 41 (54.6%) were unreported, expending the spectrum of known *PKHD1* mutations from 303 to 344. We found that the proportion of truncating mutations was around 55%, exactly as in series of patients with early demise, compared with 21–23% in series of living patients.[10,15–17,20,21] Most patients with 2 truncating mutations die in the neonatal period.[10,15,17] Overall, at least one amino acid substitution mutation is necessary for survival in the neonatal period.[21] In our series, severe genotypes, characterized by two truncating mutations, predominated in the forms of ARPKD detected relatively early by prenatal ultrasonography. However, the 16 neonates with non-severe genotype died shortly after birth, indicating that the absence of two truncating mutations cannot be regarded as synonymous of a favorable prognosis (Table 3).

Considering that most histological lesions increased with GA, it is not surprising that the intrinsic effect of mutations on histological lesions could not be evidenced when GA was not taken into account. On the other hand, after adjustment

of histological lesions to GA, severe genotype was correlated with significantly more severe extension of collecting duct dilatation in the cortex and to a lesser degree, to more severe lesions of cortical tubules than non-severe genotype (Table 4). However, the degree of proximal tubule dilatation, glomerular lesions, and cortical fibrosis, as well as the severity of portal fibrosis, adjusted to GA, were not significantly different according to the severity of the genotype (Table 4). Although the cohort of patients was relatively important, it probably was insufficient for these criteria to reach statistical significance.

Additional events may interfere with the genotype–phenotype correlations. First, as allelic variation in gene expression is common in the human genome,[31] this can modulate the level of the various *PKHD1* mutants, leading to differences in phenotypic expression. Second, modifier genes could also modulate the phenotype despite a unique *PKHD1* genotype.[32] Interactions between *PKHD1* and *HNF1B*,[33] the kinesin family member 12,[34] or *PKD1*[35] and *PKD2*,[36,37] the mouse orthologs of the genes linked to human autosomal dominant polycystic kidney disease, have been shown in animal models.

In conclusion, this study shows that the presence of two truncating mutations in *PKHD1* is associated with the most severe renal lesions in patients with ARPKD detected early during pregnancy. However, patients with late prenatal detection, less severe histological lesions and without two truncating mutations may have postnatal demise, showing the limits of genetic screening to predict the clinical outcome.

MATERIALS AND METHODS

Criteria for the inclusion in the study

Histological confirmation of the diagnosis of ARPKD/CHF was the prerequisite for inclusion. Histological criteria for ARPKD were: (i) fusiform dilatation of the collecting ducts, with a radial disposition from the calyx and medulla towards the cortex[38–40] (ii) CHF defined by portal fibrosis surrounding increased numbers of hyperplasic and ectatic biliary ducts, with normal hepatocellular histology.[39,40] In a single patient without renal histology, the diagnosis of ARPKD relied on liver histology and the presence of *PKHD1* mutations.

Patients

Patients were recruited by three French laboratories involved in the genetic diagnosis of ARPKD (Department of Genetics, Pr A. Munnich, Hôpital Necker-Enfants Malades, Paris and the laboratories of ED and L M-C).

Seventy-four patients representing 74 unrelated nuclear families were studied, including 54 fetuses (median GA at medical pregnancy termination: 28 weeks (14–37 weeks)), and 20 postnatal demise patients (median age at death, 20 days (1 day to 6 months)). Median GA for the whole cohort was 30 weeks. In the case of kindred (12 siblings, six pedigrees) or twins (four twins, two pedigrees), only the first propositus was included in the study because the comparison of histological data in the siblings of similar GA or twins did not indicate intrafamilial variability.

Father and mother's country of birth was France in 70 and 77% of cases, respectively. Other parents were of Italian (4%), Turkish (4%), Spanish (2%), Finish (1%), Algerian (1%), Lebanon (1%) or Chinese (1%) origin. Origin was unknown in 15% of parents.

Histological study

Renal specimens were available in 73 cases and liver specimens in 61, including the only patient with no renal tissue available. All specimens were collected during autopsy except one (post-mortem biopsy). They were fixed in 4% buffered formalin and embedded in paraffin. Special attention was paid for taking frontal cortico-medullary sections of the kidney passing through the hilus allowing correct analysis of cystic structures. Five micrometer-thick sections were stained with trichrome, hematoxylin eosin safran or periodic acid of Schiff. Some of the samples were examined after Red Sirius staining of fibrosis, or after immunohistochemical-specific identification of collecting ducts (using anti-EMA antibody) or proximal tubules (using CD15 antibody). They were examined independently by three pathologists (ALD, MCG, and RB) for confirmation of the diagnosis and evaluation of the severity of kidney and liver lesions. The evaluation was concordant between the 3 pathologists in all cases except 4, leading to common re-examination of the slides of these patients.

PKHD1 mutation screening

For each patient, informed consent for genetic testing was obtained from both parents. Genomic DNA was isolated from the index cases using standard procedures on the following samples: amniotic fluid, chorionic villus, lung or liver tissue obtained during the autopsy, peripheral blood. GenomiPhi HY DNA amplification kit (GE Healthcare Europe GmbH, Orsay, France) was then used for the representational amplification of the extracted DNA as a limited amount of DNA was obtained from the original samples. Mutation screening was done for the 66 exons encoding the 4074 amino acids fibrocystin protein, including splice sites and at least 20 bp of intronic sequence. Preamplified DNA was subjected to PCR using the primer sets described in Supplementary Table S5, thus generating 66 PCR fragments of 198 to 1840 bp for direct sequencing. PCR amplification was performed using Thermo-Start DNA Polymerase Mastermix from Abgene (Epsom, UK) on 30 to 60 ng of genomic DNA according to the manufacturer's recommendations. The cycling conditions were as follows: 1×15 min 95 °C; 35×30 s 95 °C, 30 s 55 °C, 2 min 72 °C; 1×5 min 72 °C. Direct sequencing was performed on both strands using the reverse and forward primers designed for amplification and the BigDye terminator chemistry on ABI 3730XL capillary sequencers (Applied Biosystems, Foster City, CA, USA). Additional sequencing internal primers were used for exons 32, 58, and 61 (Supplementary Table S5).

PKHD1 sequence analysis

Sequence alignments were done using Seqscape software (Applied Biosystems). Classification of the DNA variations was done according to the Mutation Database for ARPKD from the Aachen University (http://www.humgen.rwth-aachen.de).

Newly described variations were classified as polymorphism or mutation as follows. Silent sequence variations (intronic or exonic) were classified as polymorphism. Truncating mutations (nonsense mutations, non in-frame insertions and deletions and consensus splice mutations (Supplementary Table S6)) were classified as deleterious mutations. Finally, missense mutations were analyzed using six different tests: conservation between human, chimpanzee, dog, mouse, chicken, and frog PKHD1 protein[19] and five *in silico* protein

structure and function prediction programs (Supplementary Table S7). Sequence variation was considered as causing disease mutation when at least 50% of the performed tests predicted a pathological effect. Of the 13 unreported missense mutations, all but one (S1867G) fulfilled those criteria. However, as the S1867 is evolutionary conserved and the S1867N variation was reported as deleterious in a previous mutation study (http://www.humgen.rwth-aachen.de), we considered S1867G as a missense mutation (Supplementary Table S7).

Statistical analysis

Results were expressed as median (range) for quantitative variables and numbers (percentages) for categorical variables. Associations between categorical variables were examined with χ^2, Fischer's exact test or trend χ^2 statistics as appropriate. The study of relationships between severity of mutations and renal/hepatic lesion grades were adjusted on gestational age dichotomized at median value using Cochran–Mantel–Haenszel statistics. All tests were two-sided and values of <0.05 were considered statistically significant. All analyses were performed with SAS statistical package (SAS 9.1, SAS Inc., Cary, NC, USA).

DISCLOSURE

All the authors declared no competing interests.

ACKNOWLEDGMENTS

We thank ML Gaume (CHU Montpellier), M Peuchmaur (Hôpital Robert Debré, Paris), J Politis (CH Vannes), H Sevestre (CHU Amiens) and S Vanecloo (CH Valenciennes) for providing tissue specimens and C Antignac and A Munnich for providing some of the DNAs studied in this work. We are grateful to the anonymous reviewers for their careful and constructive suggestions. This work has been partially reported during the 40th Annual Meeting of the European Society of Paediatric Nephrology, Palermo, Italy, 7 October 2006. Supported by the Programme Hospitalier de Recherche Clinique (AOM 03 024), Délégation à la Recherche Clinique–Ile de France, Assistance Publique-Hôpitaux de Paris and the Association pour l'Information et la Recherche sur les Maladies Rénales Génétiques.

SUPPLEMENTARY MATERIAL

Table S1. Clinical and histological data.
Table S2. Mutations in *PKHD1* and corresponding genotype.
Table S3. Distinct mutations in *PKHD1*.
Table S4. Relationships between genotype, renal lesions and portal fibrosis non-adjusted to gestational age.
Table S5. Primers for PCR amplification and sequencing of *PKHD1*
Table S6. Characteristics of splice mutations.
Table S7. Characteristics of missense mutations.
Supplementary material is linked to the online version of the paper at http://www.nature.com/ki

REFERENCES

1. Zerres K, Mucher G, Becker J *et al.* Prenatal diagnosis of autosomal recessive polycystic kidney disease (ARPKD): molecular genetics, clinical experience, and fetal morphology. *Am J Med Genet* 1998; **76**: 137–144.
2. Guay-Woodford LM, Desmond RA. Autosomal recessive polycystic kidney disease: the clinical experience in North America. *Pediatrics* 2003; **111**: 1072–1080.
3. Zerres K, Rudnik-Schoneborn S, Deget F *et al.* Autosomal recessive polycystic kidney disease in 115 children: clinical presentation, course and influence of gender. *Acta Paediatr* 1996; **85**: 437–445.
4. Roy S, Dillon MJ, Trompeter RS *et al.* Autosomal recessive polycystic kidney disease: long-term outcome of neonatal survivors. *Pediatr Nephrol* 1997; **11**: 302–306.
5. Capisonda R, Phan V, Traubuci J *et al.* Autosomal recessive polycystic kidney disease: outcomes from a single-center experience. *Pediatr Nephrol* 2003; **18**: 119–126.
6. Shneider BL, Magid MS. Liver disease in autosomal recessive polycystic kidney disease. *Pediatr Transplant* 2005; **9**: 634–639.
7. Onuchic LF, Furu L, Nagasawa Y *et al.* PKHD1, the polycystic kidney and hepatic disease 1 gene, encodes a novel large protein containing multiple immunoglobulin-like plexin-transcription-factor domains and parallel beta-helix 1 repeats. *Am J Hum Genet* 2002; **70**: 1305–1317.
8. Ward CJ, Hogan MC, Rossetti S *et al.* The gene mutated in autosomal recessive polycystic kidney disease encodes a large, receptor-like protein. *Nat Genet* 2002; **30**: 259–269.
9. Ward CJ, Yuan D, Masyuk TV *et al.* Cellular and subcellular localization of the ARPKD protein; fibrocystin is expressed on primary cilia. *Hum Mol Genet* 2003; **12**: 2703–2710.
10. Bergmann C, Senderek J, Sedlacek B *et al.* Spectrum of mutations in gene for autosomal recessive polycystic kidney disease (ARPKD/PKHD1). *J Am Soc Nephrol* 2003; **14**: 76–89.
11. Wang S, Luo Y, Wilson PD *et al.* The autosomal recessive polycystic kidney disease protein is localized to primary cilia, with concentration in the basal body area. *J Am Soc Nephrol* 2004; **15**: 592–602.
12. Zhang MZ, Mai W, Li C *et al.* PKHD1 protein encoded by the gene for autosomal recessive polycystic kidney disease associates with basal bodies and primary cilia in renal epithelial cells. *Proc Natl Acad Sci USA* 2004; **101**: 2311–2316.
13. Wilson PD. Polycystic kidney disease. *N Engl J Med* 2004; **350**: 151–164.
14. Torres VE, Harris PC. Mechanisms of disease: autosomal dominant and recessive polycystic kidney diseases. *Nat Clin Pract Nephrol* 2006; **2**: 40–55.
15. Bergmann C, Senderek J, Kupper F *et al.* PKHD1 mutations in autosomal recessive polycystic kidney disease (ARPKD). *Hum Mutat* 2004; **23**: 453–463.
16. Bergmann C, Senderek J, Schneider F *et al.* PKHD1 mutations in families requesting prenatal diagnosis for autosomal recessive polycystic kidney disease (ARPKD). *Hum Mutat* 2004; **23**: 487–495.
17. Bergmann C, Senderek J, Windelen E *et al.* Clinical consequences of PKHD1 mutations in 164 patients with autosomal-recessive polycystic kidney disease (ARPKD). *Kidney Int* 2005; **67**: 829–848.
18. Sharp AM, Messiaen LM, Page G *et al.* Comprehensive genomic analysis of PKHD1 mutations in ARPKD cohorts. *J Med Genet* 2005; **42**: 336–349.
19. Losekoot M, Haarloo C, Ruivenkamp C *et al.* Analysis of missense variants in the PKHD1-gene in patients with autosomal recessive polycystic kidney disease (ARPKD). *Hum Genet* 2005; **118**: 185–206.
20. Adeva M, El-Youssef M, Rossetti S *et al.* Clinical and molecular characterization defines a broadened spectrum of autosomal recessive polycystic kidney disease (ARPKD). *Medecine (Baltimore)* 2006; **85**: 1–21.
21. Furu L, Onuchic LF, Gharavi A *et al.* Milder presentation of recessive polycystic kidney disease requires presence of amino acid substitution mutations. *J Am Soc Nephrol* 2003; **14**: 2004–2014.
22. Bergmann C, Kupper F, Dornia C *et al.* Algorithm for efficient PKHD1 mutation screening in autosomal recessive polycystic kidney disease (ARPKD). *Hum Mutat* 2005; **25**: 225–231.
23. Osathanondh V, Potter EL. Pathogenesis of polycystic kidneys. Type 1 due to hyperplasia of interstitial portions of collecting tubules. *Arch Pathol* 1964; **77**: 466–473.
24. Nakanishi K, Sweeney Jr WE, Zerres K *et al.* Proximal tubular cysts in fetal human autosomal recessive polycystic kidney disease. *J Am Soc Nephrol* 2000; **11**: 760–763.
25. Avner ED, Studnicki FE, Young MC *et al.* Congenital murine polycystic kidney disease. I. The ontogeny of tubular cyst formation. *Pediatr Nephrol* 1987; **1**: 587–596.
26. Nauta J, Ozawa Y, Sweeney Jr WE *et al.* Renal and biliary abnormalities in a new murine model of autosomal recessive polycystic kidney disease. *Pediatr Nephrol* 1993; **7**: 163–172.
27. Moyer JH, Lee-Tischler MJ, Kwon HY *et al.* Candidate gene associated with a mutation causing recessive polycystic kidney disease in mice. *Science* 1994; **264**: 1329–1333.
28. Ricker JL, Gattone 2nd VH, Calvet JP *et al.* Development of autosomal recessive polycystic kidney disease in BALB/c-cpk/cpk mice. *J Am Soc Nephrol* 2000; **11**: 1837–1847.
29. Woollard JR, Punyashtiti R, Richardson S *et al.* A mouse model of autosomal recessive polycystic kidney disease with biliary duct and proximal tubule dilatation. *Kidney Int* 2007; **72**: 328–336.

30. Nagasawa Y, Matthiesen S, Onuchic LF *et al.* Identification and characterization of Pkhd1, the mouse orthologue of the human ARPKD gene. *J Am Soc Nephrol* 2002; **13**: 2246–2258.
31. Lo HS, Wang Z, Hu Y *et al.* Allelic variation in gene expression is common in the human genome. *Genome Res* 2003; **13**: 1855–1862.
32. Rossetti S, Harris PC. Genotype-phenotype correlations in autosomal dominant and autosomal recessive polycystic kidney disease. *J Am Soc Nephrol* 2007; **18**: 1374–1380.
33. Hiesberger T, Shao X, Gourley E *et al.* Role of the hepatocyte nuclear factor-1beta (HNF-1beta) C-terminal domain in Pkhd1 (ARPKD) gene transcription and renal cystogenesis. *J Biol Chem* 2005; **280**: 10578–10586.
34. Mrug M, Li R, Cui X *et al.* Kinesin family member 12 is a candidate polycystic kidney disease modifier in the cpk mouse. *J Am Soc Nephrol* 2005; **16**: 905–916.
35. Garcia-Gonzalez MA, Menezes LF, Piontek KB *et al.* Genetic interaction studies link autosomal dominant and recessive polycystic kidney disease in a common pathway. *Hum Mol Genet* 2007; **16**: 1940–1950.
36. Kim I, Fu Y, Hui K *et al.* Fibrocystin/polyductin modulates renal tubular formation by regulating polycystin-2 expression and function. *J Am Soc Nephrol* 2008; **19**: 455–468.
37. Kim I, Li C, Liang D *et al.* Polycystin-2 expression is regulated by a PC2-binding domain of intracellular portion of fibrocystin. *J Biol Chem* 2008; **283**: 31559–31566.
38. Kissane JM. Renal cysts in pediatric patients. A classification and overview. *Pediatr Nephrol* 1990; **4**: 69–77.
39. Mac Rae Dell K, Mc Donald RA, Watkins SL *et al.* Polycystic Kidney disease. In: Avner ED, Harmon WE, Niaudet P (eds). Pediatric Nephrology, Lippincott Williams and Wilkins: Philadelphia, USA. 5th edn 2004 pp 675–699.
40. Gagnadoux MF. Cystic diseases. In: Davison AM, Cameron JS, Grunfeld JP *et al*. (eds). Oxford Textbook of Clinical Nephrology, Oxford University Press: New York, USA. 3rd edn 2005 pp 2297–2304.