http://www.kidney-international.org

see commentary on page 1421

Mutational analysis of the *RPGRIP1L* gene in patients with Joubert syndrome and nephronophthisis

MTF Wolf[1,2], S Saunier[3,4], JF O'Toole[1], N Wanner[5], T Groshong[6], M Attanasio[1], R Salomon[3,4], T Stallmach[7], JA Sayer[1,8], R Waldherr[9], M Griebel[5], J Oh[10], TJ Neuhaus[11], U Josefiak[12], C Antignac[3,4], EA Otto[1] and F Hildebrandt[1,2]

[1]Department of Pediatrics and Communicable Diseases, University of Michigan, Ann Arbor, Michigan, USA; [2]Department of Human Genetics, University of Michigan, Ann Arbor, Michigan, USA; [3]INSERM U-574, Hôpital Necker-Enfants Malades, Paris, France; [4]Université Paris Descartes, Paris, France; [5]Department of Pediatrics, Munich University of Technology, Munich, Germany; [6]Department of Child Health, School of Medicine, University of Missouri, Columbia, Missouri, USA; [7]Pediatric Pathology Unit, University Hospital, Zurich, Switzerland; [8]Institute of Human Genetics, School of Clinical Medical Sciences, University of Newcastle upon Tyne, Newcastle upon Tyne, UK; [9]Gemeinschaftspraxis Pathologie, Heidelberg, Germany; [10]Department of Pediatrics, Pediatric Nephrology, University Children's Hospital, Heidelberg, Germany; [11]Nephrology Unit, University Children's Hospital, Zurich, Switzerland and [12]Department of Internal Medicine I, Cologne-Merheim Hospital, Cologne City Hospitals, Cologne, Germany

Joubert syndrome (JS) is an autosomal recessive disorder, consisting of mental retardation, cerebellar vermis aplasia, an irregular breathing pattern, and retinal degeneration. Nephronophthisis (NPHP) is found in 17–27% of these patients, which was designated JS type B. Mutations in four separate genes (*AHI1*, *NPHP1*, *CEP290/NPHP6*, and *MKS3*) are linked to JS. However, missense mutations in a new ciliary gene (*RPGRIP1L*) were found in type B patients. We analyzed a cohort of 56 patients with JS type B who were negative for mutations in three (*AHI1*, *NPHP1*, and *CEP290/NPHP6*) of the four genes previously linked to the syndrome. The 26 exons encoding *RPGRIP1L* were analyzed by means of PCR amplification, CEL I endonuclease digestion, and subsequent sequencing. Using this approach, four different mutations in the *RPGRIP1L* gene in five different families were identified and three were found to be novel mutations. Additionally, we verified that missense mutations are responsible for JS type B and cluster in exon 15 of the *RPGRIP1L* gene. Our studies confirm that a T615P mutation represents the most common mutation in the *RPGRIP1L* gene causing disease in about 8–10% of JS type B patients negative for *NPHP1*, *NPHP6*, or *AHI1* mutations.

Kidney International (2007) **72,** 1520–1526; doi:10.1038/sj.ki.5002630; published online 24 October 2007

KEYWORDS: Joubert syndrome; nephronophthisis; *RPGRIP1L*

Correspondence: *F Hildebrandt, Department of Pediatrics and Communicable Diseases, University of Michigan, 8220C MSRB III, 1150 West Medical Center Drive, Ann Arbor, Michigan 48109-0646, USA. E-mail: fhilde@umich.edu*

Received 30 May 2007; revised 10 August 2007; accepted 28 August 2007; published online 24 October 2007

Joubert syndrome (JS (MIM 213300)) is a developmental disorder characterized by cerebellar ataxia, mental retardation, oculomotor apraxia, hypotonia, and neonatal tachypnea.[1,2] Subtypes of JS, which are termed Joubert syndrome and related disorders, include further clinical features such as nephronophthisis (NPHP), retinal dystrophy, ocular coloboma, hepatic fibrosis, occipital encephalocele, and polydactyly. The prevalence of JS is estimated as 1:100 000.[3] The 'molar tooth sign' (MTS) represents the classical neuroradiological feature, caused by cerebellar vermis aplasia, thickened and elongated superior cerebellar peduncles, and a deepened interpeduncular fossa.[4] In 17–27% of patients with JS, renal involvement with NPHP and retinitis pigmentosa (RP) is reported.[5] This subgroup of patients with JS is called JS type B or cerebello-oculo-renal syndrome patients.[6] NPHP (MIM 256100) constitutes the most frequent genetic cause of end-stage renal disease (ESRD) in children and young adults.[7] The characteristic histological findings in NPHP are renal interstitial fibrosis, interstitial cell infiltrates, and tubular atrophy with cyst development at the corticomedullary border.[7] Retinal degeneration occurs in 10–15% of NPHP patients, which results in the Senior–Loken syndrome.

Four genes are known to cause JS: *AHI1* on chromosome 6q23.3,[8,9] *NPHP1* on chromosome 2q13,[10] *CEP290/NPHP6* on chromosome 12q21.3,[11,12] and *MKS3* on chromosome 8q24.[13] Mutations in the *AHI1* gene are causative in 10–15% of patients with Joubert syndrome and related disorders,[2] who often present with retinal dystrophy. NPHP was found only in a few older subjects with *AHI1* mutations.[10,14] *NPHP1* deletions are found in approximately 25% of NPHP patients,[15] whereas *NPHP1* deletions are found only in 1–2% of all JS patients. However, neurological involvement compatible with JS type B was detected in 8.9% of all *NPHP1*-deletion carriers retrospectively.[16] In a recent publication, *CEP290/NPHP6* mutations were found in 43% of JS

type B patients and only in 2% of a cohort of JS patients without retinal involvement.[17] Mutations in *CEP290/NPHP6* were also found in patients with Leber's congenital amaurosis (LCA) and in patients with Meckel–Gruber syndrome (MKS).[18,19] Whereas LCA patients show only an ocular phenotype, MKS represents a rare autosomal recessive severe disorder, characterized by multicystic kidney dysplasia, polydactyly, and liver bile duct proliferation. Typical features of MKS such as occipital encephalocele and polydactyly were also reported in some patients with JS.[19] Mutations in the *MKS3* gene were found in MKS patients as well as in 3 out of 22 (approximately 10%) JS patients.[13] Finally, two other JS loci with yet unidentified genes were found on chromosome 9q34.3 and on chromosome 11p12–11q13.3.[20,21]

Very recently, mutations in the *RPGRIP1L* gene were found and the *RPGRIP1L* gene was identified as a new gene causing JS type B.[22] Using a homozygosity approach, a 1.6-Mb region was defined encompassing the deleted genetic region causing the $ft^{-/-}$ (fused toe) phenotype in mice. The characteristics of the $ft^{-/-}$ mouse are (i) fusion of digits 1–3 and polydactyly, (ii) an enlarged thymus, (iii) a very small mesencephalon and telencephalon with exencephaly, (iv) microphthalmia, and (v) laterality defects.[23] Only recently, cystic kidney disease was reported in the $ft^{-/-}$ mouse.[22] The central nervous system phenotype of the $ft^{-/-}$ mouse is reminiscent of the central nervous system phenotype of JS patients. The finding that *RPGRIP1L* was located within the deletion of the $ft^{-/-}$ mouse led to the discovery of *RPGRIP1L* mutations as the cause of NPHP type 8. In addition, recently, *RPGRIP1L* was shown to interact with *NPHP4*, thereby indicating a function of *RPGRIP1L* in the complex of other nephrocystins and outlining *RPGRIP1L* as a promising candidate gene for NPHP.[24]

Interestingly, a clinical overlap between JS and MKS in humans was confirmed with the discovery of the *RPGRIP1L* gene: in JS patients, missense mutations or the presence of only one truncating mutation was found, whereas two truncating mutations were identified in the more severe MKS phenotype.[22] *RPGRIP1L* encodes a protein expressed in primary cilia, which is compatible with the ciliary theory of cystic kidney disease.[22,25] The genes causative for NPHP and MKS were also shown to be expressed in renal primary cilia.[26,27] To see how frequent mutations in *RPGRIP1L* are responsible for JS type B, we analyzed a cohort of 56 patients from central Europe and the United States with JS type B, who were all negative for homozygous *NPHP1* deletions and mutations in *AHI1* and *CEP290/NPHP6*. All 26 exons encoding the *RPGRIP1L* gene were analyzed by the CEL I assay, and aberrant bands were subsequently sequenced.[15]

RESULTS

Clinical data

The five kindreds presenting with *RPGRIP1L* mutations included six living individuals affected with NPHP and neurological symptoms compatible with JS (JS type B) (Table 1). All six patients had impaired renal function. ESRD ensued between 4 and 21 years of age (Table 1). Renal biopsy was available in four out of the six patients. Brain imaging by magnetic resonance imaging (MRI) was available in three of the six patients, who all showed MTS and CVA (Table 1). One patient presented with pituitary agenesis (A1183 II-1). Mental retardation was found in all patients. Other neurological phenotypes included (i) alalia (1/6), (ii) delayed speech development (1/6), and (iii) gait problems (2/6) (Table 1). Ocular motor apraxia type Cogan was found in one out of six patients, ocular coloboma was found in one out of six patients and unspecified 'vision problems' were

Table 1 | Synopsis of the clinical data, current age, age at ESRD, extrarenal manifestations, mutation, and amino-acid change in the patients with mutation in the *RPGRIP1L* gene[a]

Family, individual	Age (years)	Ethnic origin	ESRD[b] (years)	NPHP	OMA	MR	LF	CVA	Other manifestations	Exon	*RPGRIP1L gene*, nucleotide alterations, segregation[c]	RPGRIP1L protein, effect on coding sequence[d]
A166 II-3	8	USA	4	+	–	+	–	–	Impaired vision	15	c.1843A>C, H	T615P (*Armadillo*)
F138 II-1	22	Swiss	10	+	+	+	–	+	Alalia	15	c.1843A>C, H	T615P (*Armadillo*)
F138 II-2	21	Swiss										
A762 II-3		German	10	+	+	+	–	+		15	c.1897T>C, H	C633R (*Dr*)
A1183 II-1	6	German	–	+	–	+	+	+	Postnatal tachypnea, pituitary agenesis, abdominal myofibroblastic tumor	23	IVS23-2A>G, h	Obligatory splice-site defect
F631 II-1	29	German	21	+	–	+	+	ND	Ocular coloboma	10	c.1177G>A, h (P)	E393K (*Dr*)

CVA, cerebellar vermis aplasia; ESRD, end-stage renal disease; fs, frameshift; IVS, intervening sequence; LF, liver fibrosis; MR, mental retardation; ND, not determined; NPHP, nephronophthisis; OMA, ocular motor apraxia type Cogan.
[a]All mutations were absent from >96 healthy control subjects.
[b]Age of onset of ESRD.
[c]H, homozygous; h, heterozygous; M, maternal; P, paternal; no specification if parent DNA not available for mutational analysis.
[d]Species dating back farthest in evolution, in which the amino-acid residue of the wild-type allele is conserved, *Dr, Danio rerio.*

described in one patient (A166 II-3). No retinal defects were found in five of the six patients. However, the unspecified vision problem in A166 II-3 does not exclude a retinal involvement in this patient. Liver fibrosis was described in two out of six patients (F631 II-1 and A1183 II-1). One of the six patients, A1183 II-1, developed a myofibroblastic abdominal tumor.

The clinical phenotypes are described specifically as follows:

Family A166. Patient A166 II-3 is an 8-year-old daughter of a consanguineous North American family. One of her brothers died with ESRD during infancy. She presented with ESRD at the age of 4 years. After initial resuscitation, a kidney biopsy was performed and was compatible with NPHP. Renal ultrasound (US) showed diffuse increased echotexture and loss of corticomedullary differentiation. She also presented with developmental delay (e.g., delayed speech development) and gait problems. She was able to walk at the age of 2 years. In addition, 'vision problems' were reported but were not specified by the pediatrician. Interestingly, an MRI did not show an 'MTS' in her case. Her 5 years younger brother is also developmentally delayed. He has a reduced glomerular filtration rate and polyuria. His renal biopsy was consistent with NPHP. His MRI revealed the MTS. No blood sample for molecular analysis was available in his case.

Family F138. Both affected siblings (F138 II-1 and II-2) descend from a Swiss kindred. Patient II-1 is a 22-year-old male patient who was diagnosed with JS and presented with ESRD at the age of 10 years. He required resuscitation when first symptoms of ESRD appeared. His renal biopsy showed atrophic tubules with thickened basement membranes. Moreover, the tubules showed microcysts. Interstitial inflammation was seen at the corticomedullary border (Figure 1). He received a kidney transplant from his father at the age of 11 years. Additional symptoms in sibling II-1 were cerebellar vermis hypoplasia, ocular apraxia, mental retardation, and delayed language skills. The brain MRI showed a slightly dilated fourth ventricle and a completely missing cerebellar vermis. Interestingly, no retinal defect was noted. While II-1 is able to ride a bike, his sister is affected much more severely. For example, she is unable to walk due to severe muscle hypotonia and hypotrophy. She also suffers from mental retardation. Her renal US showed hyperechogenic texture and small kidneys with multiple cysts, compatible with NPHP.

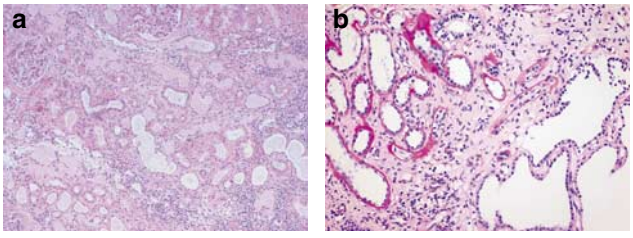

Figure 1 | Histopathology in patients with *RPGRIP1L* mutations. Histopathology from patient F138 II-1 (**a**) and patient A762 II-3 (**b**) showed the characteristic triad of NPHP with interstitial fibrosis, patchy lymphocytic infiltrate, cystic dilation of tubules, and tubular basement membrane disruption. Hematoxylin–eosin, original magnification $\times 50$.

Family A762. Patient A762 II-3 is a 12-year-old male offspring from a consanguineous German kindred. He presented with ocular motor apraxia type Cogan at the age of 4 months and with ESRD at the age of 10 years. Kidney biopsy revealed tubulointerstitial infiltrates and tubular microcysts (Figure 1b). Renal US showed no corticomedullary differentiation and hyperechogenic texture. Further symptoms were mild mental retardation, muscular hypotonia, and gait problems due to ataxia. MRI imaging of the brain was performed in the follow-up and revealed an MTS and thickened superior cerebellar peduncles, thus confirming the diagnosis of JS. Interestingly, no retinal dysfunction was reported.

Family A1183. A1183 II-1 is a 7-year-old male patient from Germany, who was diagnosed with JS. His symptoms included mental retardation, cerebellar vermis aplasia, pituitary agenesis, liver fibrosis, and impaired renal function. As a consequence of his pituitary agenesis, he has pituitary insufficiency and receives hormonal replacement therapy with growth hormone, hydrocortisone, and thyroxin. Imaging studies by MRI did not reveal any pituitary tissue in the sella. Liver fibrosis was detected by clinical symptoms of esophageal varices and was confirmed by liver biopsy. The renal US showed enlarged kidneys with hyperechogenic texture. Renal biopsy revealed focal tubular atrophy, interstitial fibrosis, and few tubular microcysts. His renal function remains stable, and he does not need dialysis. In addition, this patient developed an abdominal myofibroblastic tumor. No ocular phenotype was reported. No other family members are affected.

Family F631. F631 II-1 is a 29-year-old male patient from Germany, who presented with mental retardation, ocular coloboma, liver fibrosis, and ESRD. Liver fibrosis was confirmed by liver biopsy. No other family members are affected. Renal US revealed small hyperechogenic kidneys.

Mutation analysis

The CEL I assay was recently shown to be highly sensitive for mutational analysis by detecting 92% of known NPHP mutations (73 out of 79 mutations).[15] The strength of the CEL I mutation detection assay using celery extract is its simplicity, effectiveness, and inexpensiveness. CEL I is extremely stable during the purification process, during the storage period at -20°C, and during the whole assay. In the CEL I assay, 298 out of 1456 PCR products resulted in an aberrant band and were subsequently sequenced. In our cohort of 56 JS type B patients, we detected three missense and one splice-site mutation in five kindreds (Figure 2). In addition, two individuals of this cohort (A166 II-3 and A762 II-1) were found to be homozygous in the *RPGRIP1L* region due to 50K single nucleotide polymorphism data. In A166 II-3, the previously published c.1843A>C (T615P) mutation was identified (Figure 2a). We detected the same mutation in

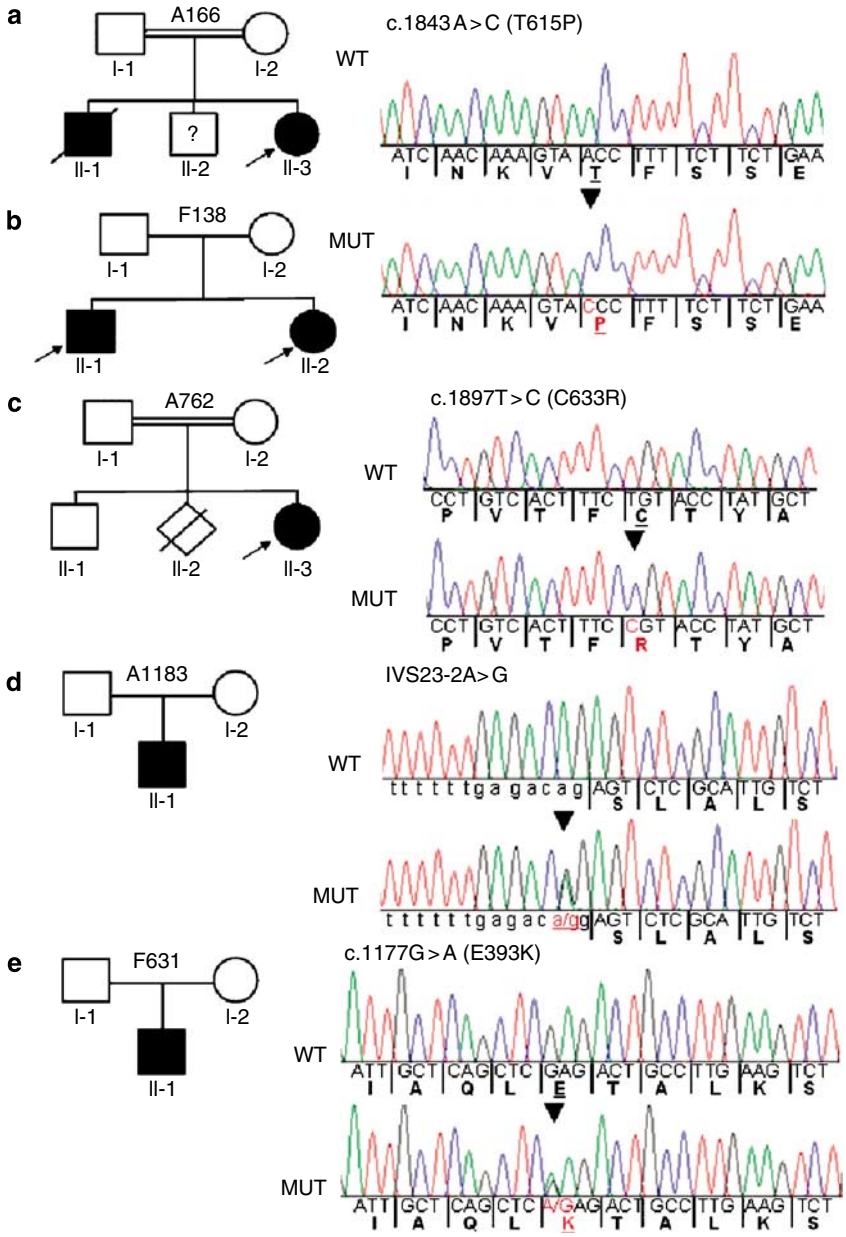

Figure 2 | Pedigrees and results of the mutational analysis in all kindreds with *RPGRIP1L* mutations. Circles denote females and squares denote males. Filled symbols denote affected individuals and crosshatches denote deceased individuals. Roman numerals denote generations. Arrows denote individuals in whom DNA was available for haplotype analysis. The upper sequence shows the wild-type (WT) sequence and the lower sequence reveals the mutation (MUT) in each family. The resulting amino acids are indicated below sequences. Mutations in (**a–c**) are located in exon 15. (**a** and **b**) In affected individuals of kindreds A166 (II-3) and F138 (II-1 and II-2), a homozygous c.1843A>C was detected resulting in a T615P amino-acid exchange. (**c**) The affected individual II-3 in family A762 showed a homozygous c.1897T>C substitution, causing a C633R change. (**d**) The affected subject II-1 in A1183 carried a heterozygous splice-site mutation IVS23-2A>G at a 100% conserved site. (**e**) The affected individual II-1 in family F631 showed a heterozygous c.1177G>A substitution, causing an E393K change. The altered nucleotides are indicated by an arrowhead, and the altered amino acid is underlined in the WT and is in red and underlined in the mutated sequence.

two affected siblings of kindred F138 (II-1 and II-2) (Figure 2b). The amino acid threonine at position 615 is evolutionarily conserved down to armadillo. In individual A762 II-1, we found a new homozygous c.1897T>C (C633R) missense mutation in exon 15 (Figure 2c). The amino acid cysteine at position 633 is evolutionarily conserved to zebrafish. The finding that mutations in A166 II-3 and A762 II-1 are homozygous is in accordance with known consanguinity. Both mutations are located in the first C2 domain of *RPGRIP1L*. We also identified two novel heterozygous mutations: (i) in A1183 II-1, an obligatory splice acceptor site of exon 23 (IVS23-2A>G) was shown to be altered (Figure 2d); and (ii) in F631 II-1, the missense mutation c.1177G>A (E393K) in exon 10 (which was

inherited from the father) was identified (Figure 2e). The splice-site mutation IVS23-2A>G in A1183 II-1 is 100% conserved. RNA of this individual is not available. Because only one mutation was found in A1183 II-1 and F631 II-1 using the CEL I assay, all other exons of *RPGRIP1L* were directly sequenced in these individuals. However, no second mutation was found. As part of the mutational analysis, 11 single nucleotide polymorphisms (SNPs) of the *RPGRIP1L* gene, which are distributed all over the gene, were analyzed. Interestingly, for A1183 and F631, all 11 SNPs appear homozygous. This may indicate a larger heterozygous deletion with hemizygosity for these SNPs. None of the mutations were found in the 100 healthy controls. Therefore, mutations detected by us are unlikely to be common polymorphisms and are most likely disease causing. Finally, using the CEL I assay, multiple new SNPs in the *RPGRIP1L* gene were identified (data available from the authors).

DISCUSSION

Very recently, mutations in *RPGRIP1L* were published as a new cause of JS type B and MKS.[22] The first publication of mutations in the *RPGRIP1L* gene lists six different mutations in five JS type B patients.[22] We performed mutational analysis in a cohort from central Europe and North America of 56 JS type B patients, using the CEL I assay to determine how frequent mutations in the *RPGRIP1L* gene in JS type B occur. By this approach, we identified four different mutations in the *RPGRIP1L* gene in five different families. Three of the four mutations were novel.

In the case of *RPGRIP1L*, a clear genotype–phenotype correlation seems to exist, with only one truncating mutation or a homozygous missense mutation causing JS type B, whereas homozygous truncating mutations cause MKS.[22] We are able to confirm that nontruncating mutations of the *RPGRIP1L* gene are disease causing for JS type B, because we identified only missense mutations but no truncating mutations.

Although the number of patients with mutations in our cohort is limited, a spectrum of different phenotypes was identified in association with mutations in *RPGRIP1L*.

Whereas developmental delay and NPHP were found in all patients, other symptoms occurred more rarely (e.g., liver fibrosis and OMA) (Table 1). Surprisingly, individual A1183 II-1 revealed pituitary agenesis, indicating a potential clinical overlap with RHYNS syndrome (retinitis pigmentosa, hypopituitarism, NPHP, and skeletal dysplasia). Interestingly, in our six patients with *RPGRIP1L* mutations, RP was described in only one. Delous *et al.*[22] also described only one *RPGRIP1L* patient with RP, whereas almost all *NPHP6* patients had RP. We confirm in our patients the rare association of RP and mutations in *RPGRIP1L*.

In three individuals, we found the previously identified c.1843A>C (T615P) mutation in a homozygous state. In the previous publication of Delous *et al.*,[22] the c.1843A>C (T615P) mutation occurred in two patients only heterozygously. By immunoprecipitation, reduced interaction of the T615P mutant protein with nephrocystin-4 was demonstrated.[22] The T615P mutation and the new homozygous C633R mutation are both located in the C2 domain of the RPGRIP1L protein, close to the nephrocystin-4-interacting region. Four of our six patients harbor homozygous mutations in exon 15. Including the previously published data, the highest number of mutations in *RPGRIP1L* are found in exon 15, with the c.1843A>C (T615P) mutation representing the most common mutation. This may indicate that the c.1843A>C (T615P) mutation is a putative founder mutation.

Surprisingly, we also detected two heterozygous mutations in two different individuals (A1183 II-1 and F631 II-1). Sequencing of all other *RPGRIP1L* exons did not reveal a second disease-causing mutation. We may have missed a second mutation that may be located in an intron. The second heterozygous mutation identified in A1183 affects a conserved splice site at IVS23-2A>G. Exon 23 encodes the RID domain (domain with homology to the RPGR-interacting domain of RPGRIP) of the RPGRIP1L protein. This mutation is highly likely to be involved in the pathogenesis of JS type B, as a 100% conserved splice site is altered, affecting an essential protein domain. Surprisingly, other authors have also reported a lack of a second mutation in up to 20% of their patients in other JS- or NPHP-causing genes like *MKS3* or *CEP290/NPHP6*.[13,17,19] A missing second mutation seems to become a more common phenomenon and may be explained by oligogenic inheritance as observed in Bardet–Biedl syndrome, which shares a significant phenotypic overlap with NPHP and JS.[28] More and more indications have accumulated for an oligogenic inheritance in JS and NPHP.[29,30] Therefore, in patients with only one mutation in *RPGRIP1L*, additional digenic mutations may be located in another disease-related gene.

The variety of disorders associated with mutations in other JS-causing genes such as *CEP290/NPHP6* is astonishing. Mutations in *CEP290* were identified to be the most frequent cause for LCA.[31] In addition, *CEP290* mutations were found in patients with JS.[11,12] Finally, *CEP290* mutations were described in patients with the severe MKS phenotype.[19] The milder LCA phenotype, affecting the eyes only, was shown to be due to the activation of a cryptic splice site in intron 26 (c.2991+1655A>G), causing low levels of wild-type transcripts from the mutant allele.[18] This hypothesis was an attempt to explain why the more severe JS phenotype did not occur in LCA individuals. However, recently, truncating mutations in *CEP290* were published in 10 families with LCA, questioning this explanation.[31] The spectrum of patients with *CEP290/NPHP6* mutations represents a clinical continuum, ranging from isolated eye disease (RP) to a potentially lethal multiorgan disorder (MKS). Surprisingly, the same variability of phenotypes (e.g., JS and MKS) can be found in patients with *RPGRIP1L* mutations, and allelism for JS and MKS was described again.[22]

Identification of the nephrocystins 1–8 by positional cloning has contributed to the unifying concept that all

proteins mutated in cystic kidney disease are expressed in primary cilia, centrosomes, or basal bodies of renal epithelial cells.[25,32] Moreover, a potential role in mechanosensing, cellular signaling, or in intraflagellar transport is discussed, as most nephrocystins are expressed in cilia.[7,33] Interaction of the AHI1 protein with nephrocystin-1 was shown previously.[34] The gene products of *MKS1* and *MKS3* were also shown to be expressed in cilia.[27] Recently, a list of over 2000 ciliary proteins in retinal epithelial cells was published containing most of the JS and NPHP disease-causing genes,[35] thus enlarging the number of potential candidate genes to a yet unexpected number.

MATERIALS AND METHODS

Patients

We obtained blood samples, pedigrees, and clinical information after receiving informed consent (http://www.renalgenes.org) from 56 patients/families with JS and NPHP (JS type B). Approval for experiments on humans was obtained from the University of Michigan Institutional Review Board. In all patients, the diagnosis NPHP was based on the following criteria: (i) clinical course with characteristic clinical signs of chronic renal failure, polyuria, polydipsia, anemia, and growth retardation; (ii) renal US or renal biopsy compatible with the diagnosis of NPHP as judged by a (pediatric) nephrologist; and (iii) pedigree compatible with autosomal recessive inheritance.

Genome-wide linkage screen

A genome-wide homozygosity mapping was performed for A166 II-1 and A762 II-1 using 50K Affymetrix SNP arrays. Data were evaluated by performing multipoint nonparametric lod scores across the whole genome using GENEHUNTER software assuming recessive inheritance. Areas of homozygosity were confirmed by haplotype analysis with microsatellite markers.

PCR amplification and sequencing

All 26 exons encoding *RPGRIP1L* were individually amplified using exon-flanking primers (sequences are available upon request). A 10-µl PCR was set up with 10 ng genomic DNA, 10 pmol each of forward and reverse primers, and 5 µl of HotStarTaqPolymerase mixture (Qiagen, Valencia, CA, USA). DNA amplification was performed on a thermal cycler (Mastercycler; Eppendorf, Hamburg, Germany, USA) using Thermo-Fast 96-well plates (ABgene, Rochester, NY, USA) and applying the same touchdown PCR protocol for amplifying all exons or microsatellite markers. The following touchdown PCR protocol was used: initial denaturation at 94°C for 15 min, followed by 20 cycles with an annealing temperature decreasing by 0.7°C per cycle, starting at 72°C for 30 s, denaturation at 94°C for 30 s, and extension at 72°C for 1 min. An additional 20 cycles were added: 94°C for 30 s, 55°C for 30 s, and 72°C for 1 min with a final extension of 72°C for 10 min. For mutational screening, 2 µl PCR products were directly used in a CEL I endonuclease digest. For sequencing, PCR products were purified using spin columns according to the manufacturer's instructions (Marligen, Ijamsville, MD, USA) and directly sequenced using the dideoxy chain termination method on an automatic capillary genetic analyzer (Applied Biosystems, Foster City, CA, USA). As part of the mutational analysis, 11 SNPs of the *RPGRIP1L* gene, which are distributed all over the gene, were documented. Eight of these SNPs were already published (rs35526952, rs4133018, rs7192060, rs2302677, rs7203525, rs2111119, and rs3213758), and three SNPs were found to be new (IVS5-29G>A, c.1351G>A, and IVS13+162G>C).

CEL I endonuclease preparation, heteroduplex formation, and CEL I treatment

Preparation of the CEL I endonuclease, heteroduplex formation, and CEL I treatment were performed as previously described by Otto *et al.*[15]

ACKNOWLEDGMENTS

We thank all members of the JS families for their participation. FH is the Frederick GL Huetwell Professor and Doris Duke Distinguished Clinical Scientist. He is supported by grants from the National Institutes of Health (NIH) (DK068306, DK064614, and DK069274). MTFW was supported by grants from the Koeln Fortune Program Faculty of Medicine, University of Cologne (184/2004); the German Kidney Fund (Deutsche Nierenstiftung); and the German Research Foundation (DFG WO 1229/2-1).

REFERENCES

1. Joubert M, Eisenring JJ, Robb JP *et al.* Familial agenesis of the cerebellar vermis. A syndrome of episodic hyperpnea, abnormal eye movements, ataxia, and retardation. *Neurology* 1969; **19**: 813–825.
2. Parisi MA, Doherty D, Chance PF *et al.* Joubert syndrome (and related disorders) (OMIM 213300). *Eur J Hum Genet* 2007; **15**: 511–521.
3. Parisi MA, Glass IA. Joubert syndrome. In: *GeneReviews at GeneTests-GeneClinics: Medical Genetics Information Resource [database online].* Copyright, University of Washington: Seattle. 1997–2006. Available at http://www.geneclinics.org or http://www.genetests.org, 2006.
4. Patel S, Barkovich AJ. Analysis and classification of cerebellar malformations. *AJNR Am J Neuroradiol* 2002; **23**: 1074–1087.
5. Valente EM, Brancati F, Silhavy JL *et al. AHI1* gene mutations cause specific forms of Joubert syndrome-related disorders. *Ann Neurol* 2006; **59**: 527–534.
6. Saraiva JM, Baraitser M. Joubert syndrome: a review. *Am J Med Genet* 1992; **43**: 726–731.
7. Hildebrandt F, Zhou W. Nephronophthisis-associated cilopathies. *J Am Soc Nephrol* 2007; **18**: 1855–1871.
8. Ferland RJ, Eyaid W, Collura RV *et al.* Abnormal cerebellar development and axonal decussation due to mutations in *AHI1* in Joubert syndrome. *Nat Genet* 2004; **36**: 1008–1013.
9. Dixon-Salazar T, Silhavy JL, Marsh SE *et al.* Mutations in the *AHI1* gene, encoding jouberin, cause Joubert syndrome with cortical polymicrogyria. *Am J Hum Genet* 2004; **75**: 979–987.
10. Parisi MA, Bennett CL, Eckert ML *et al.* The *NPHP1* gene deletion associated with juvenile nephronophthisis is present in a subset of individuals with Joubert syndrome. *Am J Hum Genet* 2004; **75**: 82–91.
11. Sayer JA, Otto EA, O'Toole JF *et al.* The centrosomal protein nephrocystin-6 is mutated in Joubert syndrome and activates transcription factor *ATF4*. *Nat Genet* 2006; **38**: 674–681.
12. Valente EM, Silhavy JL, Brancati F *et al.* Mutations in *CEP290*, which encodes a centrosomal protein, cause pleiotropic forms of Joubert syndrome. *Nat Genet* 2006; **38**: 623–625.
13. Baala L, Romano S, Khaddour R *et al.* The Meckel–Gruber syndrome gene *MKS3*, is mutated in Joubert syndrome. *Am J Hum Genet* 2007; **80**: 186–194.
14. Utsch B, Sayer JA, Attanasio M *et al.* Identification of the first *AHI1* gene mutations in nephronophthisis-associated Joubert syndrome. *Pediatr Nephrol* 2006; **21**: 32–35.
15. Otto EA, Helou J, Allen SJ *et al.* Mutation analysis in nephronophthisis using a combined approach of homozygosity mapping, CEL I endonuclease cleavage, and direct sequencing under revision. 2007.
16. Caridi G, Dagnino M, Rossi A *et al.* Nephronophthisis type 1 deletion syndrome with neurological symptoms: prevalence and significance of the association. *Kidney Int* 2006; **70**: 1342–1347.
17. Brancati F, Barrano G, Silhavy JL *et al. CEP290* mutations are frequently identified in the oculo-renal form of Joubert syndrome related disorders. *Am J Hum Genet* 2007; **81**: 104–113.
18. den Hollander AI, Koenekoop RK, Yzer S *et al.* Mutations in the *CEP290 (NPHP6)* gene are a frequent cause of Leber congenital amaurosis. *Am J Hum Genet* 2006; **79**: 556–561.

19. Baala L, Audollent S, Martinovic J *et al.* Pleiotropic effects of *CEP290 (NPHP6)* mutations extend to Meckel–Gruber syndrome. *Am J Hum Genet* 2007; **81**: 170–179.
20. Saar K, Al-Gazali L, Sztriha L *et al.* Homozygosity mapping in families with Joubert syndrome identifies a locus on chromosome 9q34.3 and evidence for genetic heterogeneity. *Am J Hum Genet* 1999; **65**: 1666–1671.
21. Valente EM, Salpietro DC, Brancati F *et al.* Description, nomenclature, and mapping of a novel cerebello-renal syndrome with the molar tooth malformation. *Am J Hum Genet* 2003; **73**: 663–670.
22. Delous M, Baala L, Salomon R *et al.* The novel ciliary gene *RPGRIP1L* is mutated in cerebello-oculo-renal syndrome (Joubert syndrome type B) and Meckel syndrome. *Nat Genet* 2007; **39**: 875–881.
23. van der Hoeven F, Schimmang T, Volkmann A *et al.* Programmed cell death is affected in the novel mouse mutant Fused toes (Ft). *Development* 1994; **120**: 2601–2607.
24. Roepman R, Letteboer SJ, Arts HH *et al.* Interaction of nephrocystin-4 and RPGRIP1 is disrupted by nephronophthisis or Leber congenital amaurosis-associated mutations. *Proc Natl Acad Sci USA* 2005; **102**: 18520–18525.
25. Otto EA, Schermer B, Obara T *et al.* Mutations in *INVS* encoding inversin cause nephronophthisis type 2, linking renal cystic disease to the function of primary cilia and left–right axis determination. *Nat Genet* 2003; **34**: 413–420.
26. Hildebrandt F, Otto E. Cilia and centrosomes: a unifying pathogenic concept for cystic kidney disease? *Nat Rev Genet* 2005; **6**: 928–940.
27. Dawe HR, Smith UM, Cullinane AR *et al.* The Meckel–Gruber syndrome proteins MKS1 and meckelin interact and are required for primary cilium formation. *Hum Mol Genet* 2007; **16**: 173–186.
28. Katsanis N, Ansley SJ, Badano JL *et al.* Triallelic inheritance in Bardet–Biedl syndrome, a Mendelian recessive disorder. *Science* 2001; **293**: 2256–2259.
29. Tory K, Lacoste T, Burglen L *et al.* High *NPHP1* and *NPHP6* mutation rate in patients with Joubert syndrome and nephronophthisis: potential epistatic effect of *NPHP6* and *AHI1* Mutations in patients with *NPHP1* Mutations. *J Am Soc Nephrol* 2007; **18**: 1566–1575.
30. Hoefele J, Wolf MTF, O'Toole JF *et al.* Evidence of oligogenic inheritance in nephronophthisis. *J Am Soc Nephrol* 2007; **18**: 2789–2795.
31. Perrault I, Delphin N, Hanein S *et al.* Spectrum of *NPHP6/CEP290* mutations in Leber congenital amaurosis and delineation of the associated phenotype. *Hum Mutat* 2007; **28**: 416.
32. Watnick T, Germino G. From cilia to cyst. *Nat Genet* 2003; **34**: 355–356.
33. Wolf MT, Lee J, Panther F *et al.* Expression and phenotype analysis of the nephrocystin-1 and nephrocystin-4 homologs in *Caenorhabditis elegans*. *J Am Soc Nephrol* 2005; **16**: 676–687.
34. Sayer JA, Otto EA, Hildebrandt F. Protein interaction partners of nephrocystin-1 using yeast-2-hybrid analysis (Abstract). *J Am Soc Nephrol* 2006; **17**: 517A.
35. Liu Q, Tan G, Levenkova N *et al.* The proteome of the mouse photoreceptor sensory cilium complex. *Mol Cell Proteomics* 2007; **6**: 1299–1317.