Evidence of Oligogenic Inheritance in Nephronophthisis

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ABSTRACT

Nephronophthisis is a recessive cystic renal disease that leads to end-stage renal failure in the first two decades of life. Twenty-five percent of nephronophthisis cases are caused by large homozygous deletions of *NPHP1*, but six genes responsible for nephronophthisis have been identified. Because oligogenic inheritance has been described for the related Bardet-Biedl syndrome, we evaluated whether mutations in more than one gene may also be detected in cases of nephronophthisis. Because the nephrocystins 1 to 4 are known to interact, we examined patients with nephronophthisis from 94 different families and sequenced all exons of the *NPHP1*, *NPHP2*, *NPHP3*, and *NPHP4* genes. In our previous studies involving 44 families, we detected two mutations in one of the *NPHP1–4* genes. Here, we detected in six families two mutations in either *NPHP1*, *NPHP3*, or *NPHP4*, and identified a third mutation in one of the other *NPHP* genes. Furthermore, we found possible digenic disease by detecting one individual who carried one mutation in *NPHP2* and a second mutation in *NPHP3*. Finally, we detected the presence of a single mutation in nine families, suggesting that the second recessive mutation may be in another as yet unidentified *NPHP* gene. Our findings suggest that oligogenicity may occur in cases of nephronophthisis.

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Nephronophthisis (NPHP; MIM 256100) constitutes the most frequent genetic cause of ESRD in children and young adults.[1] NPHP has an incidence of 1:50,000 live births in Canada and 1:1,000,000 in the United States.[2,3] Characteristic histologic findings in NPHP are renal interstitial fibrosis, interstitial cell infiltrates, and tubular atrophy with cyst development at the corticomedullary junction.[3] All NPHP variants share the described renal histology pattern with the exception of infantile NPHP (type 2), which shows additional features reminiscent of autosomal dominant or recessive polycystic kidney disease, such as kidney enlargement, absence of the tubular basement membrane irregularity characteristic of NPHP, and presence of cysts outside the medullary region. Extrarenal manifestations that are associated with NPHP include retinitis pigmentosa in Senior-Løken syndrome (SLS), ocular motor apraxia type Cogan, liver fibrosis, cone-shaped epiphyses, and coloboma of the optic nerve and cerebellar vermis aplasia in Joubert syndrome type B.[4]

NPHP is a genetically heterogeneous disorder. Six genes causing NPHP have been identified by

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positional cloning: *NPHP1* on chromosome 2q13,[1,5] *NPHP2* on 9q22,[6] *NPHP3* on 3q22,[7] *NPHP4* on 1p36,[8,9] *NPHP5* on 3q21.1,[10] and *NPHP6* on 12q21.3.[11] Approximately 25 to 30% of all patients with NPHP show large homozygous deletions of the *NPHP1* gene.[12] The gene products of *NPHP1* through *4* are known to interact.[6,7,9]

The gene product of *NPHP1*, nephrocystin-1, encodes a docking protein that interacts with components of cell–cell and cell–matrix signaling, such as p130Cas, filamin, tensin, and focal adhesion kinase 2.[13,14] It also interacts with the gene product of *NPHP4*, nephrocystin-4, mutations in which cause NPHP type 4.[9] We previously showed that *NPHP1* and *NPHP4* are strongly conserved in evolution dating back to the nematode *Caenorhabditis elegans.*[15]

We have found mutations in *inversin* to be responsible for NPHP type 2.[6] This supported a unifying pathogenic theory for cystic kidney diseases, which states that all proteins that are mutated in cystic kidney diseases in humans, mice, or zebrafish are expressed in primary cilia of renal epithelial cells. Nephrocystin-1 and inversin are located in primary cilia of renal tubular cells and co-immunoprecipitate.[6] The gene for NPHP type 3 was identified by positional cloning, and its gene product nephrocystin-3 has been shown to interact with nephrocystin-1.[7] *NPHP3* encodes a tetratricopeptide repeat domain and is expressed in the node of the developing mouse embryo.[7]

By definition, in monogenic disorders, mutations in one gene are sufficient to cause disease.[16] In the autosomal recessive disorder Bardet-Biedl syndrome (BBS; MIM209900), which shares clinical features with NPHP, mutations in more than one gene have been detected, suggesting the presence of "oligogenicity."[17,18] From more than 300 publications on NPHP occurring in siblings, the disease was assumed to be autosomal recessive. Because NPHP and BBS are phenotypically related, we examined whether oligogenicity can also be observed in NPHP by examining 94 patients for homozygous *NPHP1* deletions and mutations in all exons of *NPHP1*, *2*, *3*, and *4*. Here we demonstrate oligogenicity in patients with NPHP and suggest that in some instances, a potential third mutation might exert an epistatic effect on the phenotype by modifying the age of onset and/or the severity of the clinical phenotype.

RESULTS

Identification of Three Mutations in Two NPHP Genes

In six families with NPHP, we were able to identify three mutations in two *NPHP* genes (Table 1, Figure 1). The three mutations were distributed among the various *NPHP* genes as shown in Table 1.

Two mutations in *NPHP1* and one in *NPHP3* were detected in three kindreds. The siblings F9 II-1 and F9 II-2 showed a homozygous deletion in *NPHP1* and a heterozygous missense mutation C1189T (R397C) in *NPHP3*, which was inherited from the father. The amino acid residue R397 is conserved in evolution, including *Danio rerio* (Table 1, Figure 1). The individual F194 II-1 showed a homozygous C1756T (R586X) nonsense mutation in *NPHP1* and a heterozygous missense mutation G154A (A52T) in *NPHP3*. Segregation could not be assessed because of the lack of parental DNA (Table 1, Figure 1). The C1756T (R586X) mutation was recently published in three Italian individuals.[19] Individual F906 II-2 showed a homozygous deletion in *NPHP1* and a heterozygous missense mutation A1157G (N386S) in *NPHP3*. The amino acid residue N386 is conserved in evolution including *Xenopus laevis* (Table 1, Figure 1).

Two mutations in *NPHP1* and one in *NPHP4* were detected in one family: Both siblings F1114 II-1 and F1114 II-2 showed a homozygous deletion in *NPHP1*. However, only individual F1114 II-1 showed a heterozygous missense mutation C1532T (P511L) in *NPHP4*. The other affected sibling, F1114 II-2, who also showed the homozygous deletion in *NPHP1*, did not carry the missense mutation in *NPHP4*. Both children presented with retinitis pigmentosa. It is interesting that sibling F1114 II-1 developed ESRD at the earlier age of 9 yr, whereas his sister, F1114 II-2, entered ESRD later at age 17 yr (Table 1, Figure 1).

Combined mutations in *NPHP3* and *NPHP4* were found in two kindreds: Individual A11 II-1 showed a heterozygous frameshift mutation 435–438delAAGT (fsX148) inherited from his mother together with a heterozygous IVS24–1G→C obligatory splice site mutation in *NPHP3* inherited from his father. In addition, there was a heterozygous frameshift mutation 3364delACTG (fsX1144) in *NPHP4*, inherited from his father, resulting in a total of three putative loss-of-function mutations. This patient developed early-onset ESRD with liver disease (characterized by increased γGT and transaminases) as a result of congenital hepatic fibrosis when he was 3.5 yr of age. Renal ultrasound revealed renal microcysts. He had no retinal or neurologic involvement (normal electroretinogram at 8 yr). Both siblings, F24 II-1 and F24 II-2, showed a heterozygous IVS16–1G→C obligatory splice site mutation, inherited from the mother, and another heterozygous missense mutation G2260A (G754R) in *NPHP4*, inherited from the father. In addition, individual F24 II-1 showed a heterozygous C362G (T121R) mutation in *NPHP3*, inherited from the maternal allele, which was absent from the other affected sibling, F24 II-2 (Table 1, Figure 1).

None of the mutations described was found in 192 ethnically matched control chromosomes. Three of these families (F194 II-1, F906 II-2, and F1114 II-1 and II-2) presented with retinitis pigmentosa (Table 1). Six individuals from four kindreds presented with a homozygous deletion or mutation in *NPHP1*. Four of them showed a combination of a homozygous *NPHP1* gene deletion/mutation with a heterozygous *NPHP3* mutation (F9 II-1 and II-2, F194 II-1, and F906 II-2).

It is interesting that F194 II-1 presented with the identical nonpolar-to-polar amino acid residue exchange (A52T) of *NPHP3* previously described as a pathogenic mutation found

Table 1. Individuals who have NPHP and carry three mutations in two different *NPHP* genes[a]

Family, Individual	Ethnic Origin	ESRD (yr)[b]	Extrarenal Manifestations	*NPHP1*		*NPHP3*		*NPHP4*	
				Nucleotide Alterations, Segregation[c]	Effect on Coding Sequence[d]	Nucleotide Alterations, Segregation[c]	Effect on Coding Sequence[d]	Nucleotide Alterations, Segregation[c]	Effect on Coding Sequence[d]
F9, II-1	Germany	7	ND	Deletion, H[e]	Deletion, H	C1189T, h (P)	R397C (*Dr*)		
F9, II-2	Germany	11	ND	Deletion, H[e]	Deletion, H	C1189T, h (P)	R397C (*Dr*)		
F194, II-1	Germany	31	RP	C1756T, H[e]	R586X	G154A, h	A52T (*Hs*)		
F906, II-2	Russia	13	RP	Deletion, H[e]	Deletion, H	A1157G, h	N386S (*Xl*)		
F1114, II-1	Hungary	9	RP	Deletion, H[e]	Deletion, H	—	—	C1532T, h (P)	P511L (*Mm*)
F1114, II-2	Hungary	17	RP	Deletion, H[e]	Deletion, H	—	—	—	—
A11, II-1	France	3	LF	—	—	435–438delAAGT, h (M)[e]	fsX148	3364–3367delACTG, h (P)	fsX1144
F24, II-1	Germany	ND	ND	—	—	IVS24–1G→C, h (P) C362G, h (M)	Splice defect T121R (*Mm*)	IVS16–1 G→C, h (M) G2260A, h (P)	Splice site G754R (*Mm*)
F24, II-2	Germany	14	ND	—	—	—	—	IVS16–1 G→C, h (M) G2260A, h (P)[e]	Splice site G754R (*Mm*)

[a]All mutations were absent from >96 healthy control subjects. fs, frameshift; H, homozygous; h, heterozygous; IVS, intervening sequence; M, maternal; ND, no data available; P, paternal; RP, retinitis pigmentosa.
[b]Age of onset of ESRD.
[c]No specification of M or P 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: *Xl*, *Xenopus laevis*; *Dr*, *Danio rerio*; *Mm*, *Mus musculus*; *Hs*, *Homo sapiens*.
[e]Presence of two mutations in the same gene.

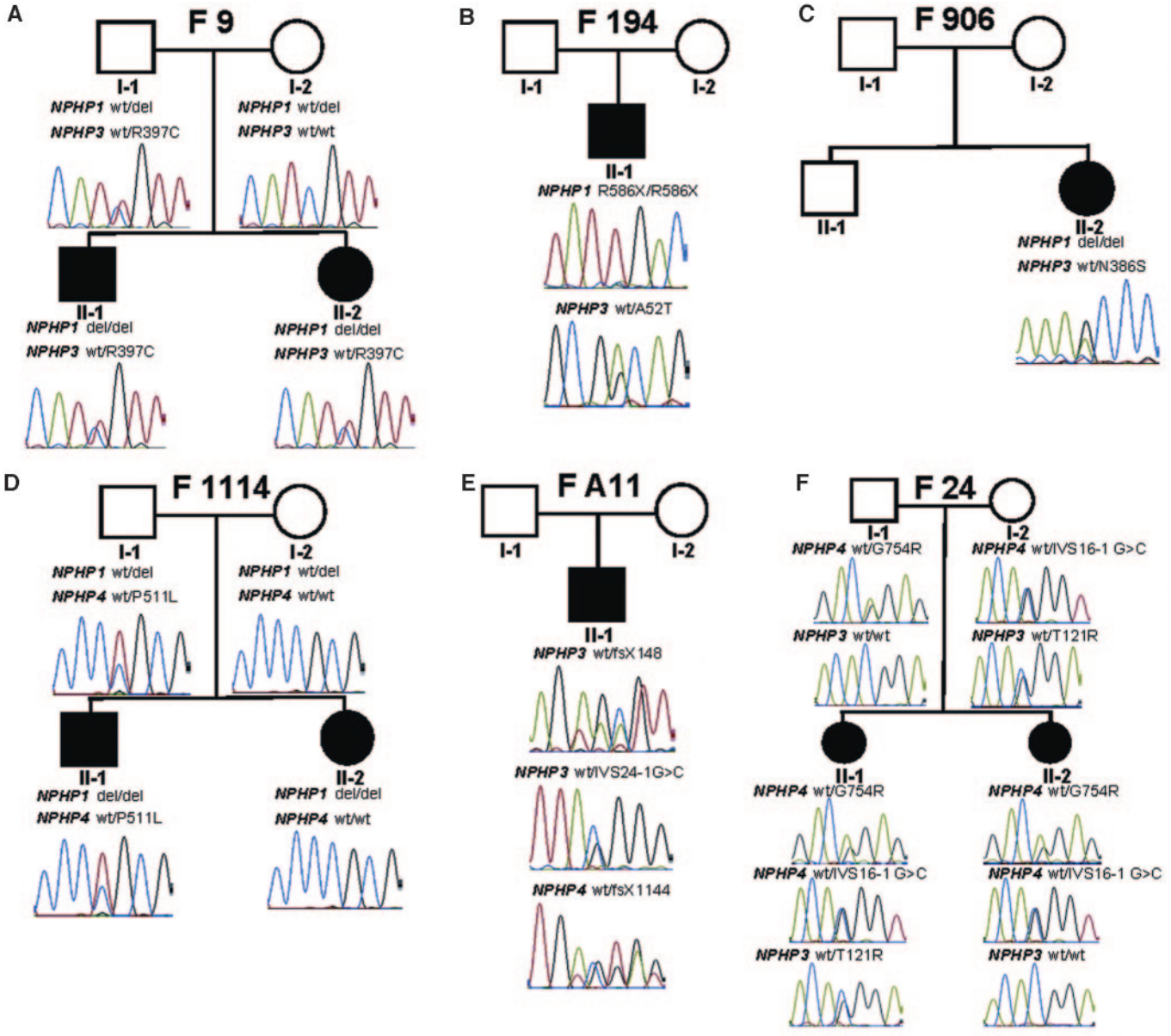

Figure 1. Oligogenic inheritance in individuals with NPHP. (A) In pedigree F9, both affected individuals II-1 and II-2 have three mutations: Homozygous deletions in *NPHP1* and one heterozygous mutation in *NPHP3* (R397C). (B) Individual F194 II-1 has a homozygous R586X in *NPHP1* combined with a heterozygous A52T mutation in *NPHP3*. (C) Individual F906 II-2 has one homozygous deletion in *NPHP1* and one heterozygous mutation in *NPHP3* (N386S). (D) In pedigree F1114, each affected individual has inherited one homozygous deletion in *NPHP1*. In addition, the earlier affected child with earlier onset of NPHP (II-1) carries a third (paternal) heterozygous mutation in *NPHP4* (P511L). (E) Individual A11 II-1 has two heterozygous mutations in *NPHP3* (fsX148 and IVS24–1G→C) and an additional heterozygous *NPHP4* mutation (fsX1144). (F) In pedigree F24, both affected individuals have two heterozygous mutations in *NPHP4* (IVS16–1G→C and G754R), and individual II-1 carries an additional heterozygous mutation in *NPHP3* (T121R). Squares denote males, circles denote females. Filled symbols indicate affected status. Sequences of the mutated nucleotide(s) are shown.

in F440 II-2 (see Supplementary Table 3).[7] The nonpolar-to-polar amino acid exchange N386S in *NPHP3* identified in F906 II-2 was also detected in patient F16 II-3 with SLS, who carried only one heterozygous mutation in all four genes (see Supplementary Table 3). All patients are offspring from nonconsanguineous marriages.

Identification of Two Heterozygous Mutations in Two NPHP Genes

We detected two heterozygous mutations in two different *NPHP* genes in kindred F281, who carried a missense mutation G1948C (A650P) in *NPHP2* and C3662T (A1221V) in *NPHP3* for patient F281 II-1 (Supplementary Table 2). The *NPHP2*

mutation of F281 II-1 is identical with the heterozygous mutation found in F443 II-1.[20] None of these mutations was found in 192 ethnically matched control chromosomes.

Identification of Single Mutations in an NPHP Gene

In nine families, we detected only one single heterozygous mutation in one of the *NPHP1* through *4* genes (Supplementary Table 3). Individuals presented with a variety of extrarenal manifestations, such as retinitis pigmentosa (F16 II-1 and II-3, F440 II-1 and II-2, and F319 II-1 and II-2), three with Joubert syndrome (F897 II-3, F394 II-7, and F700 II-6), one with ulcerative colitis (F443 II-1 and II-4), two with liver fibrosis (F390 II-1 and F443 II-1), one with biliary atresia (F616 II-2), one with Jeune syndrome (F440 II-1 and II-2), one with Cogan syndrome (F800 II-1), and one with *cor triatum sinister* and pulmonary veins dysplasia (F244 II-5). In 50 of the 94 families with NPHP examined for the *NPHP1* through *4* genes, we did not find any mutations.

DISCUSSION

NPHP is considered an autosomal recessive disease. Recent studies using positional cloning have identified six genes responsible for NPHP.[1,5–11] The gene products of the *NPHP2* through *4* genes, nephrocystin-2/inversin, nephrocystin-3, and nephrocystin-4, have been shown to interact with nephrocystin-1.[6,7,9] Recently, evidence for oligogenic inheritance was shown in BBS, which demonstrates phenotypic overlap with NPHP.[3] In some of the families with BBS the third mutation coincided with a marked increase in disease severity of specific phenotypic features.[21] Moreover, a potential epistatic effect of *NPHP6* and *AHI1* mutations in patients with Joubert syndrome and *NPHP1* mutations was recently described.[22] We therefore examined whether oligogenic inheritance would also be present in NPHP. Here we present evidence for oligogenicity in *NPHP1* through *4* by detecting two mutations in one of the *NPHP* genes in combination with a third mutation in another *NPHP* gene in six different families with NPHP (Table 1).

Given the low incidence of NPHP with approximately 1:1,000,000,[3] it seems very unlikely that the third mutations were present by chance. In a recent study, the frequency of mutations in the different *NPHP* genes was analyzed. *NPHP1* gene mutations were found in 23.4%, mutations in the *NPHP2* gene were found in 1.4%, mutations in the *NPHP3* gene were found in 0.7%, and mutations in the *NPHP4* gene were found in 2.6% of all patients with NPHP (Otto *et al.*, unpublished observations). Negative results in testing of 192 ethnically matched healthy control chromosomes supports this hypothesis, indicating that these additional third mutations are not rare polymorphisms. Especially, the heterozygous frameshift mutations in both *NPHP3* and *NPHP4* of patient A11, which result in early stop codons, together with the heterozygous obligatory splice-site mutation in *NPHP3* excludes that these changes represent polymorphisms. The number of six kindred with a total of three mutations each in two different genes is too small to draw conclusions on genotype/phenotype correlations regarding the oligogenicity. Moreover, the wide range for the age of onset of ESRD in *NPHP1* complicates genotype/phenotype analysis (7 to 25 yr of age with a median age of onset of 13 yr).[23] Whether this wide spectrum is caused by additional mutations in additional *NPHP* genes remains elusive. However, it is still worth noting that the age of onset of ESRD in F1114 is much younger in the individual with three mutations (9 yr for F1114 II-1) compared with the sibling with a homozygous deletion in *NPHP1* only (17 yr for F1114 II-2). Also, patient A11 II-1 developed ESRD earlier (at 3.5 yr) than what was reported in families with *NPHP3* or *NPHP4* mutations. In patients with *NPHP3* mutations, age of onset of ESRD has been reported between 7 and 37 yr of age with a median at 19 yr.[7]

It is interesting that the heterozygous mutations A52T and N386S in *NPHP3* have been observed each in two separate SLS pedigrees (F194 and F440, and F906 and F16, respectively) but not in healthy control subjects. Olbrich *et al.*[7] also described one patient who had SLS with a single mutation N386S in *NPHP3*. Olbrich *et al.* suggested that the unidentified second mutation may be located in a noncoding regulatory region or within an intron of *NPHP3*[7]; however, it is very suspicious that this mutation was found in three different patients with NPHP and retinitis pigmentosa. Therefore, it may be possible that this mutation modifies the clinical phenotype regarding retinitis pigmentosa. The unidentified second mutations may be present in another, yet-unidentified *NPHP* gene.

It is of note that in the report of Olbrich *et al.*,[7] three families have two mutations in *NPHP3*, whereas six families have only one mutation.[7] The high number of heterozygous single mutations found in *NPHP3* also supports that there could be a digenic or oligogenic variant of NPHP. As far as *NPHP2* is concerned, individual F281 II-1 showed two mutations, one heterozygous mutation in *NPHP2* (A650P) and one heterozygous mutation in *NPHP3* (A1221V; Supplementary Table 2). In addition, the heterozygous mutation A650P in *NPHP2* was detected in another individual (F443 II-1) but not in 192 healthy control chromosomes. Combinations of heterozygous mutations may also be envisioned as causing an NPHP phenotype, because it is known that many of these proteins interact. However, it may also be possible that this patient has two additional mutations in a yet-unknown *NPHP* gene. The notion that there exist many more unknown *NPHP* genes is further supported by the fact that in 359 of 515 children with NPHP, the causative gene is still unknown.

We identified nine families with only a single mutation in one of the *NPHP1* through *4* genes. Seven of these nine individuals show extrarenal phenotypes (Supplementary Table 3). It is interesting that the siblings in F16 show a significantly different clinical course: F16 II-3 with a single heterozygous *NPHP3* mutation showed ESRD at the age of 11, whereas her sister (F16 II-1) did not reveal this mutation and showed only mild renal impairment at the age of 20 yr. It is tempting to

speculate that onset of renal failure may be earlier in F16 II-3 as a result of the presence of this potentially pathogenic third mutation, assuming that the other causative gene has not yet been identified.

Approximately 11 yr ago, Hildebrandt *et al.*[24] already discussed two different models of genetics and pathophysiology in the NPHP complex to explain different clinical phenotypes: (*1*) A pleiotropic effect influenced by multiple allelism (*i.e.*, one mutation would lead to NPHP, whereas another mutation in the same gene would lead to NPHP with extrarenal manifestations) and (*2*) genetic locus heterogeneity, whereby different genes are responsible for the different extrarenal manifestations. Within the six known *NPHP* genes, there is a genotype/phenotype correlation described for only two of them: All cases with two *NPHP5* truncating mutations have early-onset retinitis pigmentosa (SLS).[10] All patients with two *NPHP6* truncation mutations have retinitis pigmentosa, and almost all of them have cerebellar vermis aplasia and mental retardation (Joubert syndrome).[11] However, the first model, multiple allelism, is also active in *NPHP6* mutations, whereby a hypomorphic mutation leads to Leber's congenital amaurosis only without renal or cerebellar involvement.[25]

Likewise, the existence of several different mutant alleles could explain the intrafamilial variability of our NPHP patients F1114 and F24. Intrafamilial variability may also be caused by modifier genes, which may be responsible for the occurrence of extrarenal manifestations in NPHP.[8] This situation would be similar to oligogenicity. The presence of modifier genes is supported by genetic modifications in the mouse model of *NPHP3* (*pcy* mice).[26] The identification of modifying loci was already published for *pcy*, *cpk*, *jck*, and *bpk* mouse models of human polycystic kidney disease. Modifying genes have been proposed to explain at least the diversity of polycystic kidney disease phenotypes observed in humans and animal models.[26,27] Woo *et al.*[27] defined the *pcy/pcy* mice as a mouse model for polycystic kidney disease. The loci *MOP1* and *MOP2* are described as major modifier loci that modulate the onset to ESRD. Further studies suggest that *MOP1* in mice, which also strongly modulates the progression of polycystic kidney disease in *cpk/cpk* mice,[26] corresponds to the *NPHP2* (inversin) locus in human. *MOP2* corresponds to the *NPHP3* locus in humans. These data again suggest that mutations at more than one locus may be present in at least some patients with NPHP and that there are still unidentified *NPHP* genes. The findings of many single mutations in our study and the already described single mutations in *NPHP3*[7] also indicate further genetic locus heterogeneity for NPHP.

CONCISE METHODS

Patients

After informed consent, we obtained 94 blood samples and pedigrees from patients with NPHP and their parents. All patients had the diagnosis of NPHP on the basis of the following criteria: (*1*) A clinical history consistent with NPHP, including development of ESRD, a history of polyuria, polydipsia, and anemia; (*2*) a renal ultrasound finding consistent with NPHP; and (*3*) in a subset of patients, a kidney biopsy result consistent with NPHP. In 44 families, we had already detected mutations in any one of the four *NPHP1* through *4* genes.[1,6–8] The genes involved and the clinical characteristics including the extrarenal symptoms of the previously described mutations are shown in Supplementary Table 1.1. Supplementary Table 1.2 lists the clinical characteristics of the 50 patients without a known mutation. The study was approved by the ethics committee (institutional review board) of the University of Michigan and in adherence to the Declaration of Helsinki.

Mutational Analysis

Genomic DNA was extracted from blood samples using the QIAGEN (Valencia, CA) Blood & Cell Culture DNA kit according to the manufacturer's instructions. Direct sequencing was performed for all 94 individuals in the *NPHP1* through *4* genes using the dideoxy chain termination method on an ABI Prism 3700 DNA Analyzer (Applied Biosystems, Foster City, CA). Sequences were evaluated with the Sequencher (Gene Codes, Ann Arbor, MI) software. Primers flanking 20 exons of the *NPHP1* gene, 16 exons of the *NPHP2* gene (*inversin*), 27 exons of the *NPHP3* gene, and 30 exons of the *NPHP4* gene were derived from genomic sequence (GenBank accession nos. NT_034485, NT_008470, NT_005612, and NT_028054). Primer sequences are available from the authors. For segregation analyses and screening of healthy controls for mutations, allele-specific digests, denaturing HPLC (WAVE; Transgenomics, Omaha, NE), or direct sequencing was used.

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DISCLOSURES

None.

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