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Establishing an algorithm for molecular genetic diagnostics in 127 families with juvenile nephronophthisis

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Establishing an algorithm for molecular genetic diagnostics in 127 families with juvenile nephronophthisis.

Background. Juvenile nephronophthisis (NPH1), an autosomal recessive cystic disease of the kidney, represents the most common genetic cause of end-stage renal disease in the first two decades of life. On the basis of identification of the gene (*NPHP1*) defective in NPH1 and the presence of homozygous deletions of *NPHP1* in the majority of NPH1 patients, molecular genetic diagnosis for NPH1 is now possible. Molecular genetic testing offers the only method for definite diagnosis of NPH1 and avoids invasive diagnostic measures like renal biopsy.

Methods. We examined 127 families (204 patients) with the presumed diagnosis of NPH using molecular genetic diagnostic techniques. In 68 families, renal biopsy was performed and was consistent with NPH, and in 61 families, there was more than one affected child ("multiplex families").

Results. In 74 families (115 patients), there was proof of the diagnosis of NPH1 by detection of a homozygous deletion of the *NPHP1* gene, and in 5 families a heterozygous deletion in combination with a point mutation in *NPHP1* was demonstrated. Furthermore, for 16 families, NPH1 was excluded with high likelihood by linkage analysis, and for 20 families by detection of heterozygosity for two newly identified polymorphic markers within the deletion region. In 5 of the remaining 12 families, which were noninformative for these markers, fluorescence in situ hybridization did not detect any further heterozygous deletions.

Conclusions. The diagnosis of NPH1 was proven by molecular genetic techniques in 62% of families with one or more children with the presumed diagnosis of NPH. We present evidence that there is a fourth locus for NPH, since only 6 of the 26 multiplex families in whom the diagnosis of NPH1 was excluded were compatible with linkage to other loci for NPH. On the basis of the presented data, we propose an algorithm for molecular genetic diagnostics in NPH.

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Juvenile nephronophthisis, also termed nephronophthisis type 1 (NPH1), is an autosomal recessive cystic kidney disease that constitutes the most common genetic cause of end-stage renal disease in children [1–4]. The disease leads to end-stage renal failure at a median age of 13 years [5]. For the diagnosis of NPH1, a history of polyuria, polydipsia, anemia disproportionate to the degree of renal failure, and growth retardation is indicative. Renal ultrasound shows normal kidney size, lack of corticomedullary differentiation, increased echogenicity, and later in the course of the disease cysts at the corticomedullary border of the kidneys [6–9]. As an additional diagnostic tool, renal function scintigraphy has been described showing a characteristic concentrating defect in nephronophthisis [10, 11].

Using positional cloning we recently identified the gene responsible for NPH1 (*NPHP1*) [12, 13]. Although

little is known about the pathogenesis by which a defect in *NPHP1* leads to nephronophthisis, the fact that the *NPHP1* gene product nephrocystin encodes an SH3 ("src homology 3") domain [14, 15] points toward a potential role of this gene product in protein/protein interactions, for example, in signal transduction at focal adhesions, the contact points between cells and extracellular matrix [16, 17]. In addition, it has recently been shown that nephrocystin interacts with p130Cas, which is a major component of focal adhesion signaling and has been localized also to cell–cell contacts at adherens junctions (Otto et al, unpublished observations) [18]. A defect in cell/matrix interaction would lend a potential explanation for the characteristic histologic picture of tubular basement membrane disruption in NPH and to the finding of altered integrin expression of renal tubular cells in NPH [19]. It would also be in keeping with the finding that the knockout mouse model for tensin, another component of focal adhesion signaling, histologically resembles human NPH [20].

Interestingly, in about 80% of children with NPH1, the disease is caused by deletions of the *NPHP1* gene on both parental chromosomes [5, 21]. The deletion is not due to a founder effect, but arises by homologous recombination between two copies of a 45 kb direct repeat, which flanks the *NPHP1* gene [22]. In addition, these authors have reported nonpathogenic rearrangements in patients and in controls that involved the large inverted repeats flanking the *NPHP1* gene [22]. Furthermore, we have recently cloned and characterized the shortest deletion of *NPHP1* reported thus far. It spans the region from intron 2 up to and beyond the 3′ flanking region of the gene, and occurs within a LINE1 element [23]. Homozygous deletions of the *NPHP1* region have also been described in patients with NPH and ocular motor apraxia-type Cogan (abstract; Saunier et al, *Am J Hum Genet* 61:A346, 1997) [24]. However, the question remains to why some patients present with isolated NPH1 while others suffer from NPH1 in combination with ocular motor apraxia-type Cogan, although there seems to be no distinction on a molecular genetic basis [22, 24].

Juvenile nephronophthisis (NPH1) belongs to a group of diseases [nephronophthisis/medullary cystic kidney disease (NPH/MCKD)] that share the characteristic renal histologic triad of tubular basement membrane disintegration, tubular atrophy with cyst development, and interstitial cell infiltration with fibrosis. This histologic pattern is characteristic but not pathognomonic for the NPH/MCKD group of diseases. For recessive forms, the term "nephronophthisis" is used. For isolated nephronophthisis, three different disease forms have been localized to three different loci: Juvenile nephronophthisis (NPH1) maps to chromosome 2q12-q13, and the gene has been identified [12, 15]. Infantile nephronophthisis (NPH2) is localized to 9q22-q31 and leads to end-stage renal disease in infancy [25, 26]. Since histology in NPH2 differs from the other forms of NPH, it is currently unclear whether this disease should be classified within the NPH/MCKD group of diseases. A gene locus for adolescent nephronophthisis (NPH3) maps to 3q21-q22 [56].

Recessive juvenile nephronophthisis can be associated with extrarenal organ involvement, like retinitis pigmentosa in Senior–Løken syndrome (SLS) [27, 28], with hepatic fibrosis [29], with cone-shaped epiphyses [30], or with cerebellar vermis aplasia and coloboma of the eye in Joubert syndrome type B [31, 32]. NPH has also been associated with Jeune syndrome [33–35] (asphyxiating thoraxic dysplasia), Ellis van Creveld syndrome [36], and RHYNS syndrome [37].

The second group of the NPH/MCKD group of diseases is characterized by autosomal dominant inheritance and late onset of end-stage renal failure, and is associated with hyperuricemia and gout [38, 39]. These diseases are termed "autosomal-dominant medullary cystic kidney disease" (ADMCKD). Two different loci are known so far: *MCKD1* is localized on 1q21 [40], and *MCKD2* is localized on 16p12 [41]. There is additional genetic locus heterogeneity [42–44].

Following identification of the *NPHP1* gene as responsible for NPH1, molecular genetic diagnosis can be performed by demonstration of the presence of the deletions or of point mutations in the *NPHP1* gene (www.genetests.org) [12, 15, 45, 46]. In this study, we were able to firmly establish the diagnosis of NPH1 by molecular genetic techniques in 62% of families with one or more children with the presumed diagnosis of NPH. On the basis of 127 families (204 patients) examined, we propose an algorithm for molecular genetic diagnostics in NPH. Furthermore, we present evidence that there is at least one additional locus for isolated NPH, since there were 26 multiplex families in whom we excluded the diagnosis of NPH1 with a high likelihood.

METHODS

Patients

Blood samples and pedigrees were obtained on the basis of informed consent from 204 patients with the presumed diagnosis of isolated NPH, representing 127 unrelated nuclear families. In most instances, blood samples were also available from the parents. In 68 families, the presumed diagnosis of NPH was based on a result from renal biopsy that was characteristic of NPH [47]. In the other families, the diagnosis was proposed by a (pediatric) nephrologist based on characteristic clinical signs, such as polyuria, polydipsia, anemia, or growth retardation in the presence of incipient chronic renal failure. In many instances, a result from renal ultrasound suggestive of NPH [6] was also available. In 61 families,

Table 1. Sequences of oligonucleotides used in diagnosis of NPH1

Primer name	Sequence (5′ → 3′)	Product length *bp*
146c2T-1	TGGCATTTTGGAAGTGCCTG	159
146c2T-2	TGTGAAGGCATGAGCTCTGG	
9657T-1	TCTTCACGGAGGAGCACAGTC	211
9657T-2	CCCAACTTTGCAAGCAGAAG	
Wi-18516-1	ATAGGTTGTTATTTATGACTTGGGG	109
Wi-18516-2	GAAACCCTCTGATGGCATGT	
804/6-1	TTAAGCCCAAGTAACCATAGTC	215
804/6-2	GACAAATGGAATTAACGAAATA	
146c2S-3	GTCAATCAAATGTGATACACC	133
146c2S-4b	ATGACCGGATTTTGCTGAGAG	
D2S1890-1	TTTCAGATCACCTAATGGGC	206–220
D2S1890-2	AACTGTCTGGTCGGTCATTG	
D2S1893-1	AACAAGGTGAGGCTCTGTC	244–264
D2S1893-2	TCTAAAAATGAAGCAGGATACCA	
9657T2N1	TGCATCTCTCTTCTGACTGGG	167
9657T2N2	TGTCTCTGATCAGAGGAACC	
D2S1888-1	TTTGAAGTTTGGTGTCTGTGTAA	85–97
D2S1888-2	TGAAGTCCCTTGGAAATGTT	
D2S160-1	TGTACCTAAGCCCACCCTTTAGAGC	204–218
D2S160-2	TGGCCTCCAGAAACCTCCAA	

there was more than one child affected by NPH ("multiplex families"; Table 2, group A). As recommended [48], we did not perform presymptomatic diagnosis, since no apparent benefit results for the patient at a time point in the course of NPH in which serum creatinine is still normal. Patients were excluded from the study if the presumed diagnosis was NPH in combination with extrarenal manifestations, like ocular motor apraxia-type Cogan [24], retinitis pigmentosa (Senior–Løken syndrome [27, 28]), cerebellar vermis hypoplasia/aplasia (Joubert syndrome Type B [31, 32]), hepatic fibrosis [29], Bardet–Biedl syndrome, or Jeune syndrome. The geographic origin of these families was from Europe (Austria, Estonia, Finland, France, Germany, Italy, Lithuania, The Netherlands, Sweden, Switzerland, and Turkey), North America (United States), Asia (Russia), Australia (New Zealand), India, and Africa (Togo).

Analysis for a homozygous *NPHP1* deletion

Genomic DNA was isolated by standard methods [49] directly from blood samples or from Epstein Barr virus (EBV)-transformed peripheral blood lymphocytes [50].

Analysis for a homozygous deletion of the *NPHP1* gene was performed by polymerase chain reaction (PCR) on genomic DNA of patients with NPH. One multiplex PCR was performed with two markers localized within the common *NPHP1* deletion: 146c2T-1/2 [13] and 9657T-1/2 [5]. Primer sequences are shown in Table 1. As an internal positive control marker localized outside of the deletion, we used Wi-18516-1/2 (GenBank acc. no. G24662). PCR was performed by radioactive multiplex PCR as described previously [5]. Annealing temperature in all cases was 58°C. A second multiplex PCR was performed with deletion markers 804/6 [21] and 146c2S-3/4b as an internal positive control. Our prior study of eight of the current families reported the detection of a homozygous deletion in the *NPHP1* region [5].

The absence of a large deletion in *NPHP1* does not completely exclude NPH1, since a homozygous or compound heterozygous point mutation in *NPHP1* might be present. However, statistically this situation is very unlikely because the prevalence of NPH has been estimated to be one in 1 million inhabitants [51], and most cases are caused by homozygous deletions. Thus, the allele frequency for an *NPHP1* deletion will be less than 1 in 1000. Since in this study only one chromosome with an *NPHP1* point mutation per 30 chromosomes with an *NPHP1* deletion (5 point mutations per 153 deletions) was found, the allele frequency for an *NPHP1* point mutation (if they are treated indiscriminately) would be expected to be less than 1 in 30,000. Therefore, the likelihood of two point mutations of *NPHP1* to occur together, under the assumption of Hardy–Weinberg equilibrium, would be about 1 in 900 million inhabitants. For this reason, the absence of a heterozygous deletion in *NPHP1* can be taken as exclusion with high likelihood of NPH type 1.

Haplotype analysis

Highly polymorphic microsatellite markers that localize to chromosome 2q were examined by radioactively labeled PCR followed by polyacrylamide gel electrophoresis and autoradiography, and they were scored as described previously [5]. The following microsatellite markers were employed in the haplotype analysis: D2S1890, D2S1893, 9657T2N, D2S1888, and D2S160 (order from centromere to telomere). Primer sequences are shown in Table 1. All markers were from Genethon [52] with the exception of 9657T2N. This marker is a polymorphic CA repeat marker, which we newly identified within the 3′-flanking sequence of the *NPHP1* gene by direct sequencing of PAC clone 146c2. For polymorphic marker 9657T2N, the 5′ nucleotide of primer 9657T2N1 is located 1785 bp centromeric and primer 9657T2N2 is located 1952 bp centromeric of the start codon of the first exon of the *MALL* (or *BENE*) gene [12].

Screening for point mutations in *NPHP1*

Mutational analysis by single-strand conformational polymorphism analysis (SSCP) was performed for all 20 NPHP1 exons as described [12]. Direct sequencing of SSCP products was done after purifying PCR-products with QIAquick spin columns (Qiagen, Hilden, Germany) and sequenced directly on an automated fluorescent sequencer.

Fluorescence in situ hybridization

Fluorescence in situ hybridization (FISH) was performed with P1-related artificial chromosome (PAC)

clones as described [53, 54]. The positive control probe PAC 9681, which is localized outside of the *NPHP1* deletion, was biotinylated and detected via streptavidin-conjugated fluorescein. PAC probe 146c2, which is localized within the *NPHP1* deletion, was labeled with digoxigenin and detected via rhodamine-conjugated antidigoxigenin.

Screening for a heterozygous *NPHP1* deletion

Two PCR primer pairs that we had used previously to amplify *NPHP1* exon 7 and intron 9 [12] were found to exhibit a diallelic polymorphism on SSCP. For the exon 7 polymorphism, primers EX7F and EX7R were employed as described previously [12]. The polymorphism involves a silent nucleotide exchange 654A→G with relatively equal distribution of both alleles in the population examined. This base exchange can be detected by abolishing a *Tfi* I restriction site. For intron 9, primers EX10F and EX10R were used as described previously [12]. The polymorphism involves a IVS9-47T→C exchange with relatively equal distribution of both alleles in the population examined. Both polymorphisms are positioned within the large deletion described in NPH1. Therefore, if an affected child is heterozygous for at least one of the two polymorphic markers, a large heterozygous deletion can be excluded. In some patients, only one allele was seen in both markers. In this case, we also examined the parents to determine whether there was parental noncontribution for one allele, demonstrating the presence of a heterozygous deletion or whether the marker was noninformative in this family.

RESULTS

A total of 127 families with suspected NPH in at least one member was enrolled in the molecular genetic diagnostic study on NPH1. For 68 families, the diagnosis of NPH had been confirmed by renal biopsy. In 61 families, there was more than one child affected with NPH ("multiplex families"), amounting to a total of 204 patients within the study (Table 2, group A).

Analysis for homozygous *NPHP1* deletion

Since the presence of a homozygous deletion of the *NPHP1* gene can be taken as proof for the diagnosis of NPH1 [12, 21, 22], we performed as a first diagnostic step deletion detection by PCR using three markers from within the deletion, together with two control markers from outside the deletion region, as described in the **Methods** section. We detected a homozygous deletion in the affected children of 74 families (30 with renal biopsy result), 32 of whom were multiplex families, representing a total of 115 patients (Table 2, group B). Thus, as a result of deletion analysis, the diagnosis of NPH1 was firmly established in 58% of all families enrolled. If only families with a biopsy-proven diagnosis were counted, this number was 44%, which hinted that a certain number of the families with renal biopsy result available might represent a form of NPH other than type 1 (Table 2, group B). At this point of the diagnostic work-up, 53 families, 29 of whom were multiplex, remained in the study (Table 2, group B).

Haplotype analysis

Since linkage analysis can be employed to exclude (although not prove) the diagnosis of NPH1 [5], we performed haplotype analysis for linkage to the *NPHP1* locus in the remaining 29 multiplex families. By haplotype analysis, 16 multiplex families (12 biopsy proven) were excluded from linkage to *NPHP1*, representing 38 patients (Table 2, group C). These patients most likely suffer from a form of isolated NPH other than type 1. This appears even more likely, since later some of these families were found to be compatible with linkage to *NPHP3* (Omran et al, personal communication). Since none of the patients enrolled entered end-stage renal disease within the first three years of life, infantile nephronophthisis (NPH2) can also be excluded. At this point of the diagnostic work-up, 37 families (26 with renal biopsy result and 13 multiplex) remained in the study, representing 51 patients (Table 2, group C). Among these, we expected a significant number to harbor a heterozygous deletion, since the initial analysis for a homozygous *NPHP1* deletion was positive in 44% of all families with a renal biopsy result compatible with NPH. Therefore the affected children of the 37 remaining families were screened by SSCP of all 20 *NPHP1* exons as described [12], and direct sequencing performed if the SSCP pattern suggested a point mutation.

Screening for point mutations in *NPHP1*

Two families with novel point mutations were detected that occurred hemizygously in combination with a heterozygous deletion: In family F41, the affected child carried a maternally inherited heterozygous deletion of *NPHP1* in combination with a paternally inherited point mutation in exon 9 (Fig. 1). The mutation (1024G→A) leads to a nonconservative G342R exchange, affecting an amino acid, which is conserved in nephrocystin of mouse [14] and dog [18], but not in *C. elegans* [14]. In addition, this nucleotide exchange affects an 80% conserved splice donor consensus [55] and thus most likely interferes with correct splicing. This point mutation was not seen in 70 healthy controls. The results from family F41 are shown for SSCP and direct sequencing in Figure 1 A and B, respectively.

In the affected child of family 275, in exon 6 we detected an insertional mutation (548insA), which induces a frame shift, leading to an in-frame STOP codon four amino acids downstream (D187X; Fig. 2). The mother

Table 2. Diagnostic work-up using molecular genetics in 127 families with presumed isolated juvenile nephronophthisis (NPH1)

Group	Results from diagnostic tests for NPH1 (work-up proceeds from top to bottom)	Molecular genetic diagnosis of NPH1		Families [a](histology) [b][multiplex] and patients remaining in the diagnostic work-up for NPH1	[c]Cumulative number of families with molecular genetic proof of NPH1 [a](histology) in %
		Excluded	Proven		
		Families [a](histology) [b][multiplex] patients	Families [a](histology) [b][multiplex] patients		
A	Total families enrolled			127 (68)	
		—	—	[61]	—
				204	
B	Homozygous *NPHP1* deletion detected		74 (30)	53 (38)	74 (30)
		—	[32]	[29]	58% (44%)
			115	89	
C	Linkage to *NPHP1* excluded by haplotype analysis in multiplex families	16 (12)		37 (26)	
		[16]	—	[13]	—
		38		51	
D	Heterozygous deletion with heterozygous point mutation detected by direct sequencing		5 (4)	32 (22)	79 (34)
		—	[3]	[10]	62% (50%)
			8	43	
E	Large heterozygous deletion excluded, since patient heterozygous for exon 7 or intron 9 polymorphism	20 (15)		12 (7)	
		[8]	—	[2]	—
		29		14	
F	Large heterozygous deletion not excluded, since patient homozygous for exon 7 or intron 9 polymorphism			12 (7)	
		—	—	[2]	—
				14	
G	Large heterozygous deletion excluded by FISH	5 (2)		7 (5)	
		[2]	—	[0]	—
		7		7	

[a] Number of families in whom renal histology was performed and compatible with NPH
[b] Number of "multiplex" families (i.e., with more than 1 affected child)
[c] Cumulative number of families, in whom the diagnosis of NPH1 was established in the course of molecular genetic diagnostics

was heterozygous, and the affected child was hemizygous for this point mutation, since he inherited a paternal deletion. This insertional mutation was absent from the healthy control and from the father, who heterozygously carried a deletion of this region. It was also absent from 70 healthy controls. The results from SSCP and direct sequencing are shown for family 275 in Figure 2A and B, respectively. In addition, in two families, we detected two base changes within intron 16 (IVS16 + 19C→T and IVS16 + 38T→A; data not shown). Although these base changes were not detected in 70 healthy individuals, they most likely represent innocuous polymorphisms, since they are not part of a conserved splice consensus. We have previously described three additional point mutations in *NPHP1* in combination with a heterozygous deletion [12]. Therefore, the two novel point mutations described here add up to a total of five families (4 biopsied) found within the cohort examined (Table 2, group D).

In the *NPHP1* point mutations described, it was possible to delineate clearly the parental origin of the heterozygous deletion from SSCP and direct sequencing data by the absence of one parental allele in the affected child (Figs. 1 and 2). However, we sought to demonstrate directly the presence of the heterozygous *NPHP1* deletion by FISH in families in which material was available for preparation of metaphases. As an example, Figure 3 shows the result from FISH in family F40, in whom we have previously described the combination of a maternal hemizygous point mutation (IVS14 + 1delG; Fig. 4) with a paternal heterozygous deletion [12]. Together with the three hemizygous point mutations described previously [5], five hemizygous point mutations were detected within the 37 families studied by SSCP.

Analysis for heterozygous *NPHP1* deletion

Detection of homozygous *NPHP1* deletions in 74 families and of heterozygous deletions with hemizygous point mutations in five families (4 with renal biopsy result, 3 multiplex) has lead to the definite diagnosis of NPH1 in 79 families (Table 2, group D). At this point of the diagnostic work-up, 32 families (22 with renal biopsy result), 10 of which were multiplex, remained to be classified by molecular genetic diagnostic techniques (Table 2, group D).

Since all deletions in NPH1 described so far extend across the *NPHP1* gene at a minimum from exon 3 to exon 20 [12, 22], two newly detected di-allelic SSCP polymorphisms were employed, one in exon 7 and one in intron 9 (**Methods** section), to exclude the presence of a heterozygous deletion in the remaining families. In 20 families (15 with renal biopsy result, 8 multiplex), the affected child was heterozygous for at least one of the two polymorphisms. Since in the case of a heterozygous deletion the parental noncontribution of one allele would have to be found, the state of heterozygosity in the affected child excluded the presence of a heterozygous

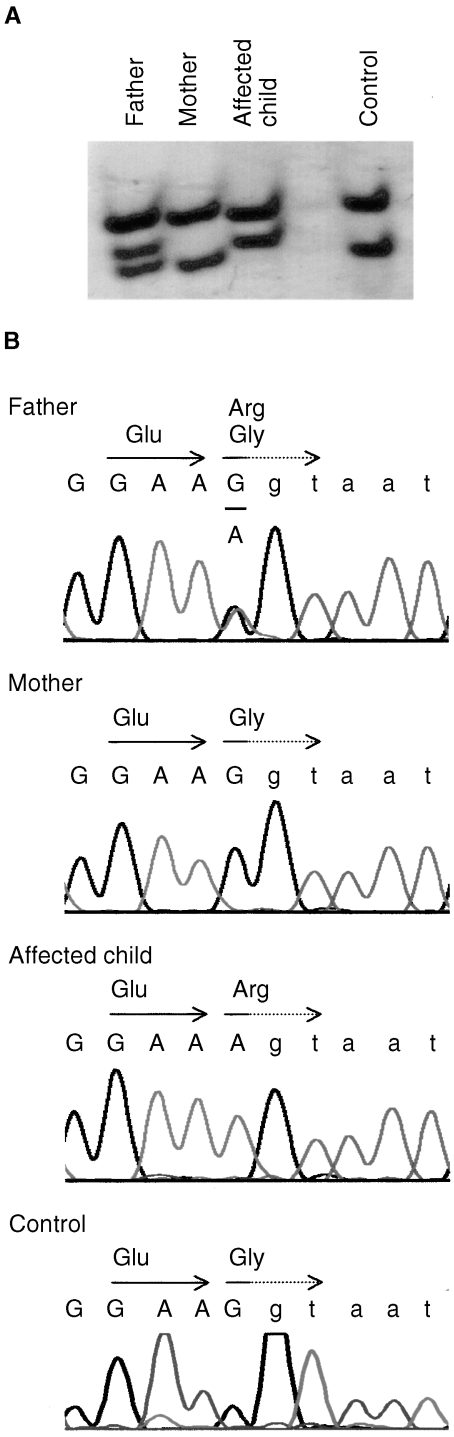


Fig. 1. Point mutation in exon 9 of the *NPHP1* gene in family F41. (*A*) SSCP shows a normal pattern in a healthy control and in the mother, who carries on one chromosome a deletion of this genetic region. In the father, there is a band shift resulting from a point mutation. This band shift occurs hemizygously in the child, since no product results in this child from the maternal chromosome, which carries the deletion. (*B*) Direct sequencing of the exon 9 splice donor junction reveals heterozygously in the father a G1024A exchange, which changes the codon (G_*splice*_gt) for a conserved glycine at position 342 into the codon (A_*splice*_gt) for an arginine, and in addition affects an 80% conserved splice donor consensus. This G1024A exchange is absent in the healthy control and in the mother, who carries a deletion of this region in one chromosome. The affected child is hemizygous for this point mutation, since he inherits a maternal deletion. Exonic nucleotides are shown as capitals and intronic nucleotides as lower case letters.

NPHP1 deletion in these families (Table 2, group E). Because the chance that two compound heterozygous point mutations are found in NPH is very low, nephronophthisis type 1 is excluded with great certainty in these families. The facts that in 15 of these families renal histopathology was consistent with NPH and that 8 families had multiple affected children corroborate the diagnosis of NPH, rendering it very likely that these patients suffer from a form of isolated NPH other than type 1.

Following exclusion of a heterozygous large deletion of the *NPHP1* gene in 20 families (29 patients), in whom the affected children were heterozygous for one of the two polymorphic markers, 12 families (7 with renal biopsy result, 2 multiplex) remained for diagnostic work-up (Table 2, group F). In these families, the affected children carried only one allele of the exon 7 and intron 9 polymorphisms, respectively, and no parental noncontribution could be demonstrated in these children. Since this finding cannot be discerned from noninformativity for the marker, NPH1 could neither be excluded nor proven for these families.

In five of these families, blood samples of EBV-transformed lymphocytes for FISH were available. We therefore examined the affected children for a heterozygous *NPHP1* deletion by FISH analysis in the same way as described for family F40 (Fig. 3). However, no heterozygous deletion was detected in any of the five families (2 with renal biopsy result, 2 multiplex), making it very likely that these patients suffer from a form of isolated NPH other than type 1 (Table 2, group G). Finally, seven families (5 biopsy proven, no multiplex), in whom no material for FISH was available, remained unsolved.

From these data, we conclude that molecular genetic diagnosis is a useful tool to prove or exclude with high likelihood the presumed diagnosis of NPH1. The fact that in our cohort there were 26 multiplex families in whom NPH1 was excluded with high likelihood, while only six were compatible with linkage to *NPHP3*, indicates that there is at least one additional locus for NPH.

DISCUSSION

In this study of 127 families with presumed isolated NPH (68 with renal biopsy result, 61 multiplex), we were able to prove the diagnosis of NPH1 by demonstrating a homozygous deletion in 74 families (58%) or by detecting a heterozygous deletion in combination with a point mutation in five families (4%), amounting to a total of 79 families (62%). In 41 families (32%; 29 with renal biopsy result, 26 multiplex), we excluded NPH type

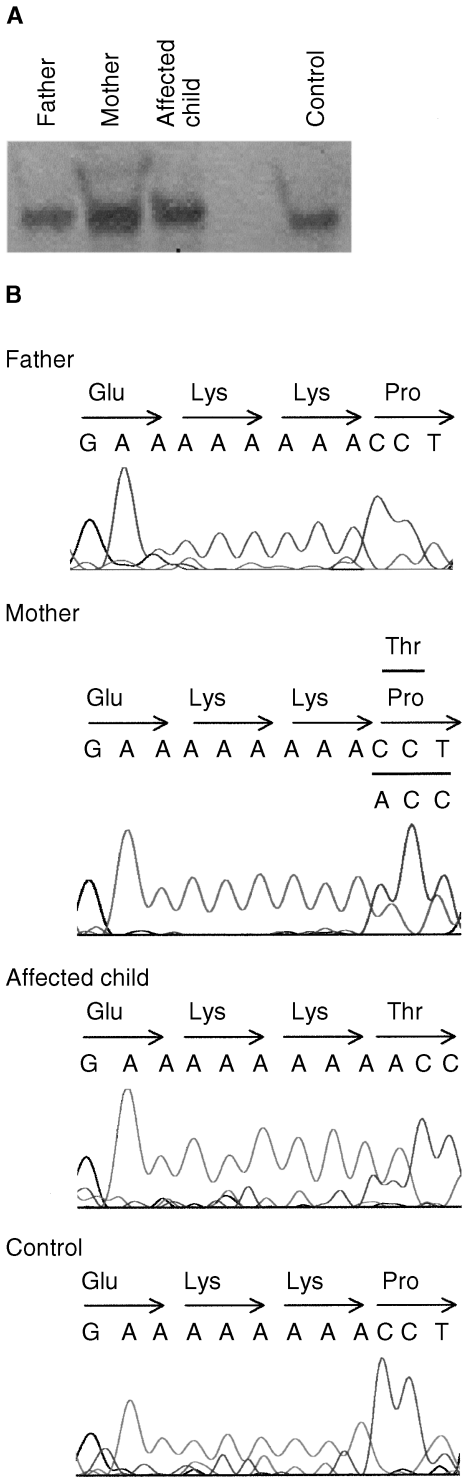


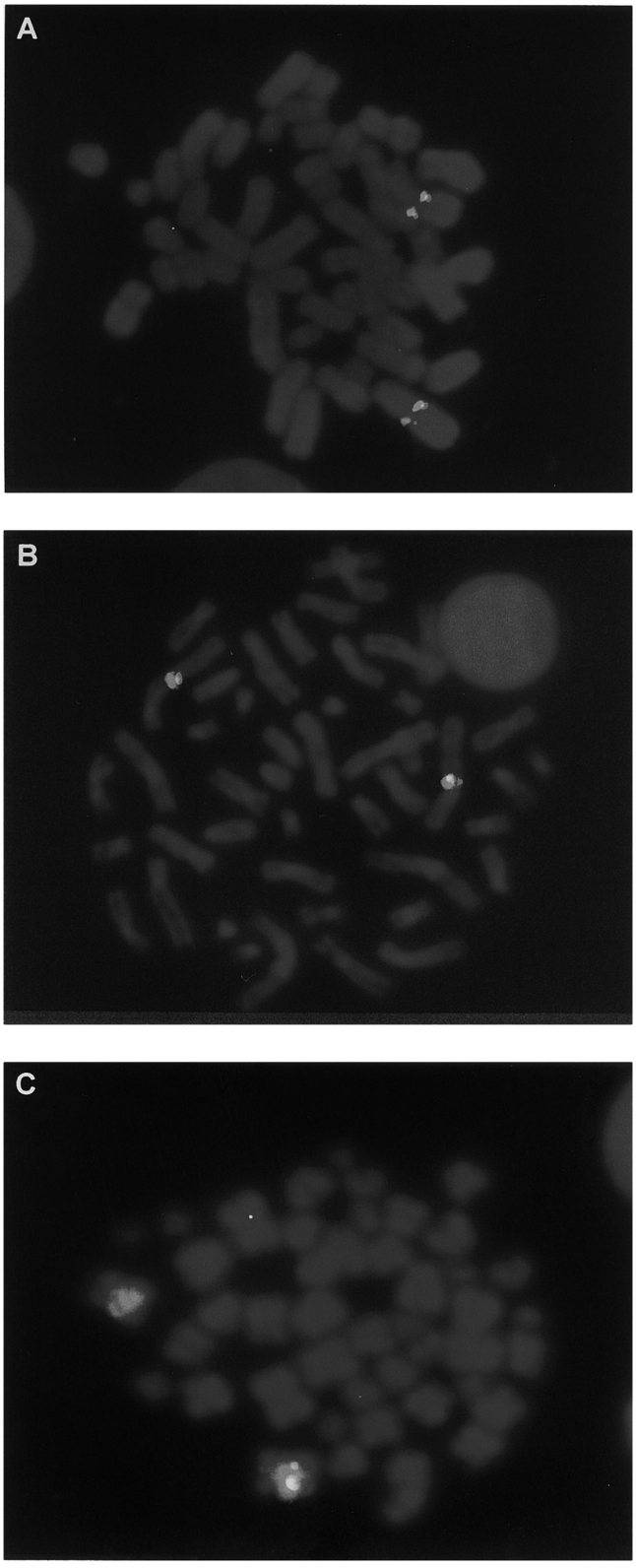
Fig. 2. Point mutation in exon 6 of the *NPHP1* gene in family F275. (*A*) SSCP shows a normal pattern in a healthy control and in the father, who carries on one chromosome a deletion of this genetic region. In the affected child, there is a band shift to a slightly longer band, which is seen heterozygously in the mother. The aberrant band occurs hemizygously in the child, since no product results in this child from the paternal chromosome, which carries the deletion. (*B*) Direct sequencing of exon 6 reveals heterozygously in the mother an insertional mutation (548insA), which induces a frame shift, leading to an in-frame STOP codon four amino acids downstream (D187X). The affected child is hemizygous for this point mutation, since he inherits a paternal deletion. This insertional mutation is absent from the healthy control and from the father, who carries a deletion of this region in one chromosome.

1 by demonstrating the absence of a linkage to *NPHP1* or excluded it with high likelihood by demonstrating the absence of a heterozygous deletion. Seven families (6%) remained unsolved. To our knowledge, this represents the largest study on molecular genetic diagnostics in NPH reported to date.

Percentage of families with NPH1

Formerly, the only method to discern NPH1 from non-NPH1 was linkage analysis. Since linkage can only be assessed in multiplex families or in families with consanguinity, only kindred fulfilling these criteria can be used for comparison between studies. In addition, in small families, linkage is only suggestive for the diagnosis but does not prove the diagnosis. Konrad et al have previously described large-scale rearrangements in 80% of the patients belonging to inbred or multiplex NPH1 families and in 65% of the sporadic cases [21]. In a previous study, we have shown compatibility with linkage to *NPHP1* only in 10 out of 16 families with NPH (62%) [5]. For the study presented here, if only multiplex and consanguineous families are considered, there was a ratio of 16 out of 68 (24%) families in whom linkage to *NPHP1* was excluded with high likelihood. Thus, the ratio of families that were compatible with linkage to *NPHP1* (76%) is again lower than the ratio of 80% of families with deletions of the *NPHP1* locus described for familial cases by Konrad et al [21].

Since this study extended molecular genetic diagnostics to sporadic cases by means of detection of heterozygous deletions and/or point mutations in *NPHP1*, we were able to assess the number of all families, regardless of the presence of multiple or single affected individuals. The diagnosis of NPH1 was excluded in 41 of 127 families (32%), and vice versa the diagnosis was confirmed in 79 of 127 (62%), while 7 (6%) were inconclusive. These data demonstrate that 68% of all families examined at most represent NPH1, while about one third of all families must harbor a genetic form other than NPH1. This finding is novel and somewhat surprising. However, if only the group with the most stringent diagnostic criteria is considered, that is, families in which data from renal biopsy were compatible with NPH, this finding is confirmed. In this group, the diagnosis of NPH1 was confirmed by molecular genetic techniques only in 34 out of 68 families (50%), while it was excluded with high likelihood in 29 out of 68 families (43%). The relatively



low percentage of homozygous deletions detected might also be due to the fact that histopathology was not evaluated by a single pathologist.

The 16 multiplex families in whom NPH1 was excluded by linkage analysis (Table 2, group C) exhibit three strong diagnostic criteria for NPH: (*1*) history and clinical signs compatible with NPH, (*2*) renal histology characteristic of NPH in 12, and (*3*) familiarity in all of them. Since linkage to *NPHP1* was excluded in these families, they must represent families with a form of isolated NPH other than type 1. In addition, there were eight multiplex families in whom no homozygous or heterozygous deletion of *NPHP1* was found (Table 2, group E), and two multiplex families in whom no heterozygous deletion was found by FISH (Table 2, group F). Thus, we identified 26 families altogether (20 with renal biopsy result) in whom NPH type 1 was excluded with a high likelihood. Only six of these families were compatible with linkage to *NPHP3* (Omran et al, personal communication). The median age for onset of ESRD in these six families was 20 years (range 11 to 20 years), which is compatible with the described median age of 19 years in NPH3 [56]. Since no cases of NPH2 were included in the study, we therefore consider it quite likely that at least one additional locus for isolated NPH exists.

Point mutations in *NPHP1*

The mutations found in this study, together with all mutations described so far in patients with NPH1, are summarized in Figure 4 in relationship to a map of putative secondary structure of the *NPHP1* gene product nephrocystin. Seven additional potential loss-of-function point mutations in combination with a heterozygous *NPHP1* deletion have been reported previously. We have described maternally-derived point mutations in exons 2, 14, and 18 [12]; Saunier et al described mutations in exons 9 and a point mutation in exon 10 [15], and Caridi et al have described point mutations in exons 15 and 17 [46].

The point mutations in exons 2, 6, and 17, and within

Fig. 3. FISH analysis for a heterozygous *NPHP1* deletion in family F40. PAC clone 9681 (fluorescein stained) is localized outside of the deletion region and was used as a positive control. It yields a signal on both chromosomes 2q12-q13. PAC clone 146c2 (rhodamine stained) is localized within the *NPHP1* deletion and therefore is absent in case of a deletion. (*A*) The healthy control carries no heterozygous *NPHP1* deletion and therefore yields a signal with both PAC clones on both homologous chromosomes 2. (*B*) The mother carries a heterozygous point mutation and therefore yields a signal with both PAC clones on both chromosomes 2 [12]. (*C*) The affected child inherits a point mutation from the mother but a deletion from the father. Therefore, a signal for PAC clone 146c2 is seen only on the maternal chromosome of this affected child, while the positive control PAC 9681 yields a signal on both chromosomes 2.

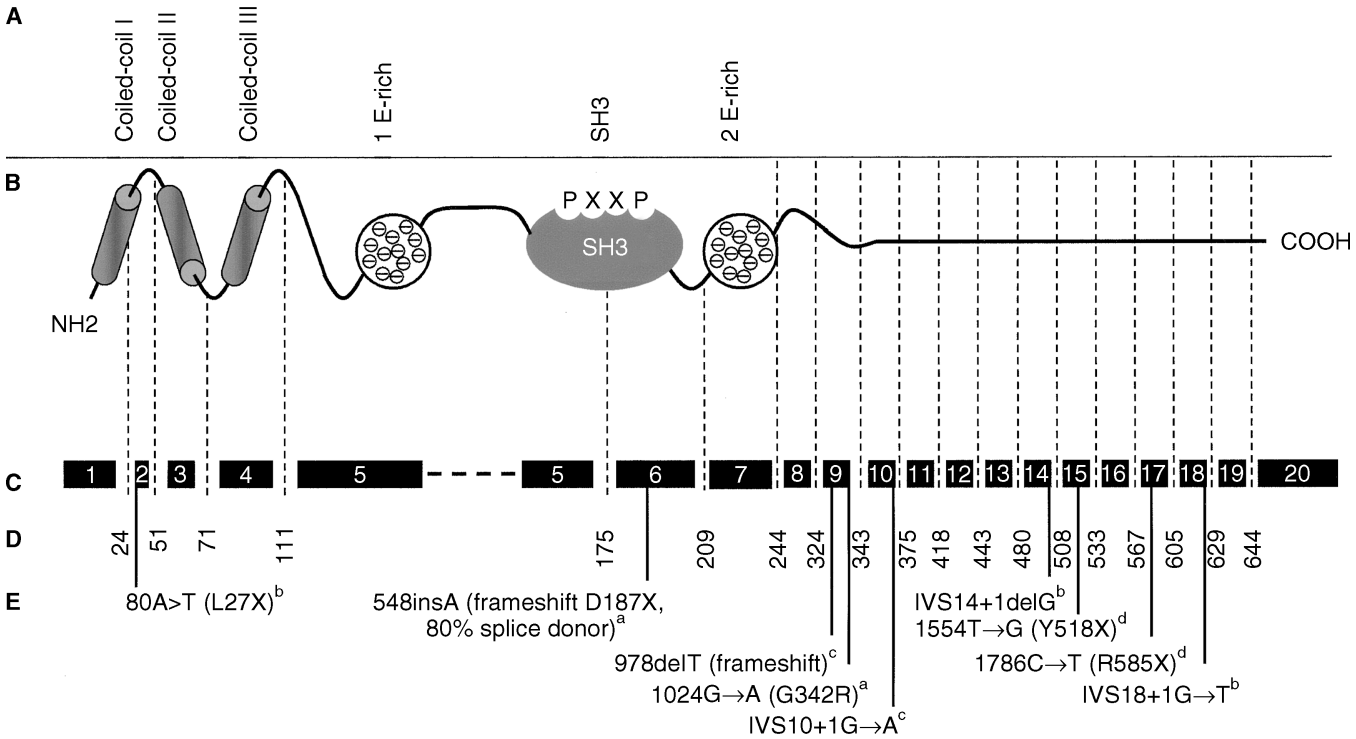


Fig. 4. Model of secondary structure for nephrocystin, the product of the *NPHP1* gene. (*A*) Putative domains of nephrocystin as derived from database searches [14]. (*B*) Graphical representation of putative nephrocystin domain structure. "PXXP" denotes SH3-binding consensus sequence. (*C*) Exon-intron boundaries in human *NPHP1* in relationship to putative domain structure in human (connected by vertical dashed lines). (*D*) Human amino acid sequence numbering in relationship to exons. (*E*) Summary of all point mutations in *NPHP1* described so far. All are found hemizygous in combination with a heterozygous deletion in patients with NPH1. Positions in relationship to exons are denoted by vertical lines. Point mutation nomenclature is used as recommended [57]. Nucleotide and amino acid numbering is in accordance with GenBank accession No. NM_000272, which is shorter by one amino acid when compared with accession No. AF023674 because of the lack of one glutamine codon at amino acid position 313. [a]This study. [b]From Hildebrandt et al [12]. [c]From Saunier et al [15]. [d]From Caridi et al [46].

introns 10, 14, and 18 were of maternal origin. The exon 9 mutation was of paternal origin, while the point mutations in exons 15 [46] and 9 [15] occurred de novo in the affected child. All of these point mutations occurred as hemizygous point mutations in combination with a heterozygous deletion. Currently, no patient with two homozygous or two compound heterozygous point mutations in *NPHP1* has been described. This is expected given the relatively small number of point mutations described to date. It is interesting to note that all point mutations found in *NPHP1* are very likely loss-of-function mutations, since they involve nonsense mutations, obligatory splice site mutations, and missense mutations leading to nonconservative amino acid exchanges. Their localization is distributed over the different domains of nephrocystin.

All point mutations have been found to be unique to only one family with the exception of the 1024G→A substitution (G342R). We have previously found this mutation hemizygously and derived from the maternal allele in a patient with NPH1 and ocular motor apraxia-type Cogan syndrome [24]. [In this publication, the same mutation was denoted 1027G→A substitution (G343R) according to an old nucleotide and amino acid numbering; note Fig. 4 legend]. The question remains unanswered as to why some patients show the symptoms of ocular motor apraxia in addition to the clinical phenotype of NPH1, despite that there seems to be no distinction on a molecular genetic basis [22, 24].

Diagnostic algorithm

A major problem in the management of patients with suspected juvenile nephronophthisis is the difficulty of firmly establishing the correct diagnosis, based on the fact that symptoms are relatively mild and renal ultrasonographic findings and even histology are characteristic but not pathognomonic [47]. In this study, a number of noninvasive diagnostic tests requiring a blood sample only are described, which provide novel tools for establishing or confirming the diagnosis of NPH1. With the help of such tests, the notorious problem of establishing a correct diagnosis can be relieved at least for the cases with deletion-positive NPH1, which constitute about two thirds of all NPH patients. From the experience with molecular genetic diagnostics in the 127 families described, we propose an algorithm for the diagnostic

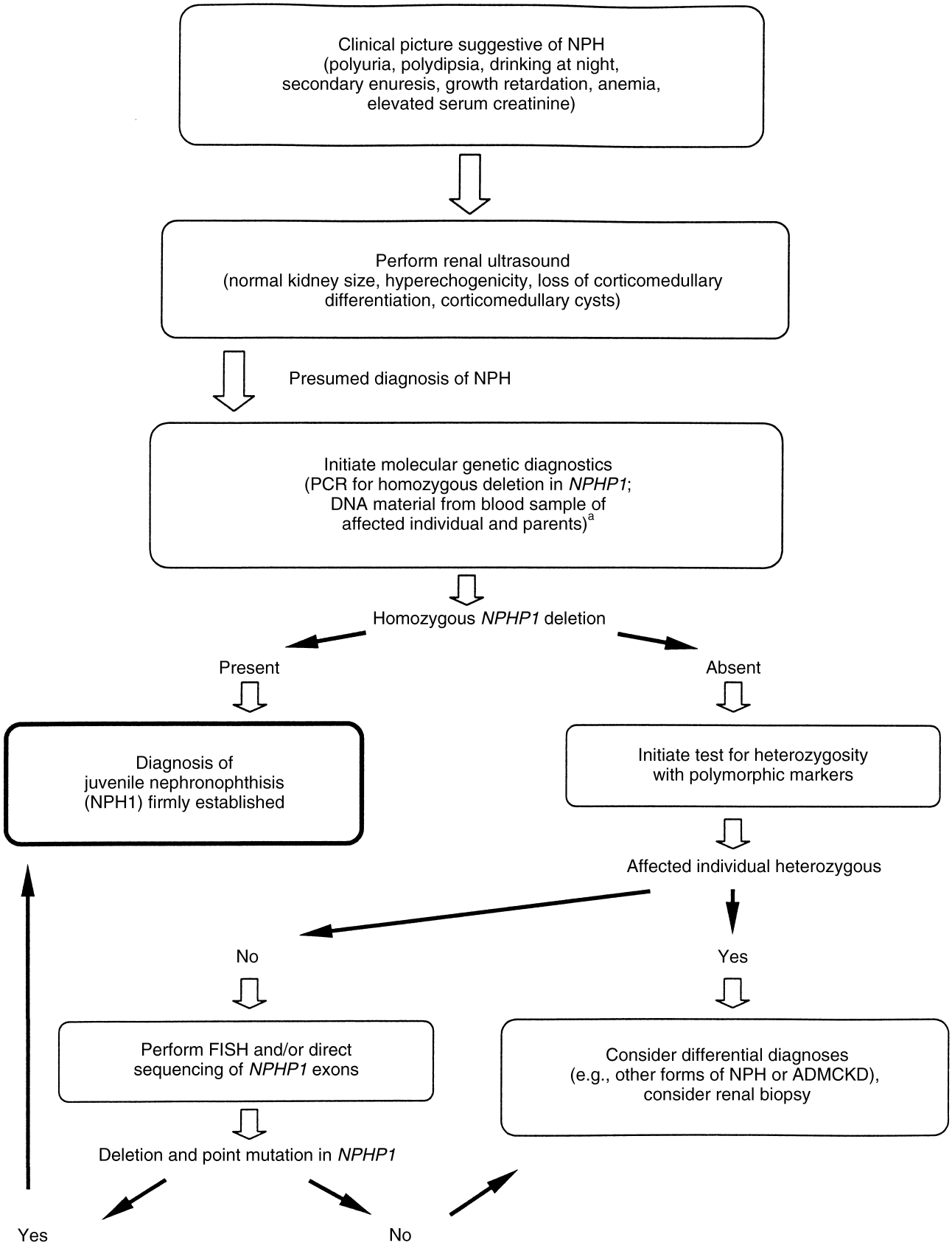


Fig. 5. Algorithm for molecular genetic diagnostics in NPH. [a](*www.genetests.org*).

work-up of patients with the presumed diagnosis of NPH. This algorithm is shown in detail in Figure 5. This molecular genetic approach, as opposed to the invasive procedure of renal biopsy, allows definite diagnosis of NPH1 in case a deletion is found. According to our presented data, this can be expected in about two thirds (62%) of all cases with suspected NPH.

As recommended [48], we refrained from performing presymptomatic diagnosis, since no apparent benefit results for the patients at a time point in the course of NPH where serum creatinine is still normal. In asymptomatic siblings of children with proven NPH1, yearly creatinine measurements are recommended in order to detect the development of renal failure early enough to initiate supportive therapy, that is, treatment of anemia, renal bone disease, growth retardation, and adequate salt and water intake. At this symptomatic stage, molecular genetic testing as a confirmation of the diagnosis is warranted.

The unusual deletion of *NPHP1* that we described previously can be detected by across-breakpoint PCR [23]. However, the common deletion of *NPHP1* occurs within a 45 kb direct repeat [22] and is, therefore, unfortunately not accessible to across-breakpoint PCR as a diagnostic procedure. To detect homozygous *NPHP1* deletions, PCR yields close to 100% specificity and sensitivity; that is, the diagnosis of NPH1 or Cogan syndrome with NPH1 is proven unequivocally if a homozygous deletion is demonstrated. In this case, a diagnosis using PCR is much safer and less invasive than renal biopsy. However, the absence of a homozygous deletion does not exclude a diagnosis of NPH1. To detect a heterozygous deletion, molecular genetic diagnostics have a limited practicality because FISH is costly and labor intensive, and frequently the polymorphic markers used here were uninformative. This method of parental noncontribution also requires the use of parental DNA and is more useful to exclude heterozygous deletions rather than to prove the existence of heterozygous deletions. An alternative is deletion detection by pulsed-field gel electrophoresis, which is a very specific but also labor-intensive method [22]. Therefore, the generation of new more highly polymorphic markers within the deletion region is needed. Haplotyping with a number of such highly polymorphic markers from the *NPHP1* deletion region performed in one PCR-based test would serve three diagnostic purposes at the same time: (*1*) detection of a homozygous deletion, (*2*) demonstration of parental noncontribution in case of a heterozygous deletion, and (*3*) haplotype analysis for linkage to *NPHP1* in case no homozygous or heterozygous deletion is found. Development of such a test would significantly improve the quality and efficiency of molecular genetic testing for NPH1.

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