**Clinical Characterization and NPHP1 Mutations in Nephronophthisis and Associated Ciliopathies: A Single Center Experience**

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**Abstract**

Nephronophthisis (NPHP) is a recessive disorder of the kidney that is the leading genetic cause of end-stage renal failure in children. Egypt is a country with a high rate of consanguineous marriages; yet, only a few studies have investigated the clinical and molecular characteristics of NPHP and related ciliopathies in the Egyptian population. We studied 20 children, from 17 independent families, fulfilling the clinical and the ultrasonographic criteria of NPHP. Analysis for a homozygous deletion of the NPHP1 gene was performed by polymerase chain reaction on the genomic DNA of all patients. Patients were best categorized as 75% juvenile NPHP, 5% infantile NPHP, and 20% Joubert syndrome-related disorders (JSRD). The mean age at diagnosis was 87.5 + 45.4 months, which was significantly late as compared with the age at onset of symptoms, 43.8 ± 29.7 months (P < 0.01). Homozygous NPHP1 deletions were detected in six patients from five of 17 (29.4%) studied families. Our study demonstrates the clinical phenotype of NPHP and related disorders in Egyptian children. Also, we report that homozygous NPHP1 deletions account for 29.4% of NPHP in the studied families in this cohort, thereby confirming the diagnosis of type-1 NPHP. Moreover, our findings confirm that NPHP1 deletions can indeed be responsible for JSRD.
Introduction

Nephronophthisis (NPHP) is an autosomal-recessive cystic kidney disease that constitutes the most frequent genetic cause of end-stage renal disease (ESRD) in the first three decades of life.\textsuperscript{1-3} The NPHP–medullary cystic kidney disease (NPHP–MCKD) complex describes a distinct clinico-pathologic entity of inherited diseases that lead to chronic renal failure on the pathologic basis of a chronic sclerosing tubulointerstitial nephritis.\textsuperscript{4}

Three clinical forms of NPHP have been distinguished based on the age at onset of ESRD: infantile,\textsuperscript{5} juvenile,\textsuperscript{6} and adolescent NPHP,\textsuperscript{7} which manifest with ESRD at the median ages of one, 13, and 19 years, respectively. NPHP can be associated with retinitis pigmentosa (Senior-Loken syndrome), liver fibrosis, cerebellar vermis aplasia [Joubert syndrome (JBTS)], and ocular motor apraxia type Cogan.\textsuperscript{8-11}

Because of the mild nature of symptoms and the lack of edema, hypertension, or urinary tract infections, there is often a delay in the diagnosis of NPHP.\textsuperscript{12} The most useful imaging technique in NPHP is ultrasonography. Kidneys are of normal or moderately reduced size and show increased echogenicity, loss of cortico-medullary differentiation, and, in later stages, cyst formation at the cortico-medullary border.\textsuperscript{13} Renal histology reveals a characteristic triad of tubular basement membrane thickening and disruption, interstitial infiltration and fibrosis, and tubular atrophy and dilatation, with or without cyst formation.\textsuperscript{14}

Molecular genetic analysis is the only diagnostic procedure by which the diagnosis of NPHP-1, NPHP-2, or NPHP-3 can be made with certainty. However, due to the presence of additional loci for NPHP, the lack of detection of mutations in the \textit{NPHP1} gene does not exclude the diagnosis of NPHP. If molecular genetic diagnostics do not detect a molecular defect, the diagnosis of NPHP can be based on the combined results of typical clinical history with polyuria, polydipsia and anemia; the classical appearance of the kidney on ultrasound and renal histology.\textsuperscript{14} The appropriate diagnosis of NPHP is important not only for anticipating progressive renal failure but also for the implications on genetic counseling.

Twelve genes have been implicated in NPHP: \textit{NPHP1,\textsuperscript{15,16} NPHP2/INVS,\textsuperscript{17} NPHP3,\textsuperscript{18} NPHP4,\textsuperscript{19,20} NPHP5/IQCB1,\textsuperscript{21} NPHP6/CEP290,\textsuperscript{22,23} NPHP7/GLIS2,\textsuperscript{24} NPHP8/\textit{RPGRIP1L,\textsuperscript{25-27} NPHP9/NEK8,\textsuperscript{28} TMEM67,\textsuperscript{29} TTC21B,\textsuperscript{30} and XPNPEP3.\textsuperscript{31}} Recently, massively parallel re-sequencing mutation analysis of 18 different NPHP-associated ciliopathy genes in 120 ascertained patients led to the identification of 57 mutated alleles, comprising 43 different mutations in 30 unrelated patients.\textsuperscript{32}

Homozygous deletions in the \textit{NPHP1} gene account for approximately 21\% of all NPHP cases, whereas the other genes contribute to less than 3\% each. Interestingly, through positional cloning, many of the causative mutations have been mapped to genes involved in centrosome and cilia function. This had contributed to a unifying theory that defines cystic kidney diseases as “ciliopathies” based on the finding that all proteins mutated in cystic kidney diseases of humans or animal models are expressed in the primary cilia or centrosomes of renal epithelial cells.\textsuperscript{33}
Little is known about the clinical characterization of NPHP and associated ciliopathies in the region, let alone the genetic molecular data on children afflicted with these diseases. This study was conducted to characterize the clinical phenotypes of infants and children with NPHP, whether isolated (only renal affection) or in the context of a complex ciliopathy (with one or more extra-renal associations). Additionally, we investigated the prevalence of \textit{NPHP1} mutations among the study group.

**Patients and Methods**

**Patients**

Children with a clinical diagnosis of NPHP, referred to the Center of Pediatric Nephrology and Transplantation, Cairo University, over a period of one year (early 2008–early 2009) were enrolled in this study. The clinical features of NPHP include presentation in the first two decades of life with symptoms of polyuria and polydipsia and signs of growth retardation and anemia. Renal ultrasound evaluation in NPHP demonstrates increased echogenicity, small to normal kidney size, and loss of cortico-medullary differentiation or small cysts at the cortico-medullary junction. Kidney biopsy, considered characteristic of NPHP, includes tubular atrophy with tubular basement membrane disruption, interstitial cellular infiltrates with fibrosis, and microcyst development.

We categorized the studied patients according to their clinical phenotype. The majority of enrolled patients had isolated NPHP, but a subset of patients had extra-renal manifestations, which included retinal degenerative changes, ocular motor apraxia, cerebellar vermis aplasia, and hepatic fibrosis (Table 1). Individuals were examined for recessive \textit{NPHP1} mutation irrespective of the presence or absence of their extra-renal manifestations. Informed consent was obtained from the parents of affected children. This study was approved by the Institutional Review Board at Cairo University Children’s Hospital.

History taking included two generation family pedigree, age at onset of symptoms (polyuria, polydipsia, and secondary enuresis), age at onset of ESRD and renal replacement therapy (RRT), if any, as well as any visual or neurological symptoms. Clinical assessment included physical growth (height and weight centiles) and blood pressure (BP) measurement. Anomalies or signs of extra-renal involvement were documented. All patients had full ophthalmo-logic evaluation to rule out ocular motor apraxia and retinal degeneration.

Laboratory investigations included hemoglobin level, urine analysis, blood urea nitrogen (BUN), serum creatinine, and calculation of the glomerular filtration rate (GFR) at the time of presentation using modified Schwartz formula.\textsuperscript{34,35}

**Imaging**

Abdominal ultrasonography was done for proper assessment of renal size, echogenicity, cortico-medullary differentiation, and to detect the presence of renal cysts, if any.\textsuperscript{13} It was also done to rule out congenital hepatic fibrosis as an extra-renal association (General Electric, Vivid 3 Pro, SyncMaster 591S device with a 3.5–7 MHz probe). All cases were examined by the same operator to avoid inter-observer variability. Magnetic resonance
imaging (MRI) of the brain was performed in the subset of patients with clinical extra-renal neurological involvement to demonstrate the distinctive “molar tooth sign.”

**Histopathology**

Renal biopsy specimens were obtained, following informed parental consent, from eight of 20 patients. Kidney biopsy was not done in the remaining 12/20 patients as a result of medical contraindications (small renal size and/or uncontrollable hypertension) or parental decline to consent the procedure.

**Mutational analysis**

Genomic DNA was extracted from blood samples collected in EDTA tubes using the QIAGEN Blood and Cell Culture DNA kit according to the manufacturer’s instructions (Qiagen, Valencia, CA, USA). Polymerase chain reaction (PCR) for mutation analysis was performed in the Hildebrandt laboratory, Ann Arbor Michigan. Cost was defrayed from the laboratory’s research funds. All individuals were screened for homozygous *NPHP1* deletions, the most common cause of NPHP. Analysis for a homozygous deletion of the *NPHP1* gene was performed by a multiplex PCR approach on genomic DNA of patients described earlier. Three pairs of primers amplifying three different exons of the *NPHP1* gene (exons 5, 7, and 20) were PCR amplified in a single PCR reaction together with two control primer pairs from another gene (*LHX9*) from chromosome 1. Primers against two exons of *LHX9* were used as internal controls to test for the presence of sufficient DNA and PCR accuracy. The primer sequences (5′ > 3′) used for the analysis are as follows: *NPHP1*-Exon5-Forward, CACTCATAGCTGGTCTGTTCTTG; *NPHP1*-Exon5-Reverse, CAGGTGTACAGGCAGAGTTTTC; *NPHP1*-Exon7-Forward, TGTTTTTACTGGAGGGTTAGGTG; *NPHP1*-Exon7-Reverse, CAGGTGTACAGGCAGAGTTTTC; *NPHP1*-Exon20-Forward, AATGGCACCCTCCATCCTAC; *NPHP1*-Exon20-Reverse, AATCGTGAGGATCCATCTCG; *LHX9*-Exon4-Forward, ATATGGCTCTGCTTTCTTC; *LHX9*-Exon4-Reverse, TGGGCAACAACTCCTTGC; *LHX9*-Exon6-Forward, ACCCTAAAAGCCAAGTTGC; and *LHX9*-Exon6-Reverse, CCTAATAGTGTCTTTGTCCTAC. One PCR reaction was set up by mixing 8 μL water (PCR-grade, 10 μL HotStarTaq® Master Mix (Qiagen), 0.1 μL of each primer (10 uM each), and 1 μL DNA (100 ng/μL). The following touchdown PCR protocol was used:

Initial denaturation: 94°C for 15 min; 24 cycles with an annealing temperature decreasing 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 one min; addition of 24 cycles using a fixed low-annealing temperature of 55°C for 30 s and denaturation at 94°C for 30 s and extension at 72°C for one min; final extension was at 72°C for 10 min. About 10 μL of the PCR reaction was electrophoretically separated on a 1.5% agarose gel for 90 min at 150 V. Lack of amplification products of all three *NPHP1* exons, was considered as a homozygous deletion in *NPHP1*. 

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**Data Analysis**

Numerical data were expressed as median and range. *P*-value less than 0.05 was considered significant.

**Results**

Twenty children from 17 families with renal findings of NPHP were included in this study. Sex distribution among the affected patients showed a slight preponderance of females, with a ratio of 1.2:1 (11 females and nine males). Seventy-five percent (15/20) were the products of consanguineous marriages. It is notable that the percentage of affected siblings was strikingly high, 65% (13/20 patients) and, likewise, the percentage of sibling death due to NPHP, which accounted for 40% (8/20) of the patients. This finding, together with the high degree of consanguinity, strongly suggests an autosomal-recessive mode of inheritance (Table 1).

While the median age of onset of symptoms in patients was 48 months (range 3–108 months), the median age at diagnosis was significantly delayed at 108 months (range 5–168 months) (*P* <0.01).

Fifteen of 20 patients (75%) presented with signs of ESRD. All the patients suffered from anemia and growth retardation when they first came to medical attention. Nineteen of 20 patients (95%) had a history typical of NPHP, with symptoms of polydipsia, polyuria, and secondary enuresis (Table 1). Four of 20 patients (20%) were hypertensive, with elevated blood pressure above the 95th percentile for age, gender, and height.

Clinically, the study patients were best categorized as: 13/20 (65%) patients with isolated juvenile NPHP and 3/20 (15%) patients as infantile NPHP, while the remaining 4/20 (20%) patients had extra-renal associations and, hence, were clinically categorized as JSRD, namely cerebello–oculo–renal syndrome (CORS).

We found extra-renal symptoms in 20% (4/20) of the study patients with neurological (mental retardation, ataxia, and MTS) and ophthalmologic (retinal degenerative changes and OMA) manifestations as the most frequently reported (Table 2). These four patients were categorized as syndromic juvenile NPHP, and three of them had ESRD at the time of diagnosis (age 84–168 months), whereas the fourth was defined as chronic kidney disease (CKD) stage III at the age of 84 months.

Homozygous deletions in the *NPHP1* gene were identified in six patients from five independent families out of the 17 studied families (29.4%). Five patients had isolated NPHP, whereas the sixth patient was among the JSRD group (Figure 1) with the unique MTS detected in his brain MRI images (Figure 2). The clinical and genetic characteristics of all the study patients are demonstrated in Table 3.
Discussion

In this report, we studied a cohort of 20 patients with NPHP. The common criterion in the study patients was the renal involvement. Of this cohort, six patients had positive homozygous deletion of *NPHP1* gene; five children had isolated NPHP and one patient had JSRD or CORS.

Of the 16 patients with isolated NPHP, five patients had homozygous deletion of *NPHP1* gene. Interestingly, four of these patients were clinically diagnosed as juvenile NPHP, whereas the fifth patient had the classical phenotype of infantile NPHP (NPHP type-2). This patient showed most of the clinical and histopathological features of NPHP type-2 previously reported in the literature,\(^4,17,37\) and had been reported recently as the first patient with the clinical phenotype of infantile NPHP, yet with documented homozygous deletion of the *NPHP1* gene.\(^38\)

Infantile NPHP, frequently caused by mutations in the *NPHP2/inversin* gene, differs from the other types of NPHP by the early age of onset of ESRD, usually less than five years in all reported cases, whereas the median age of ESRD in juvenile NPHP (NPHP type-1 or type-4) is about 13 years.\(^17\) Renal cortical microcysts is another criterion where detailed analysis of a murine model of NPHP2/inversin demonstrates cystic dilatation of Bowman’s capsule, proximal tubule, thick ascending limb, and collecting duct.\(^39\)

Co-occurrence of extra-renal findings was observed in four of the study patients (20%), two of whom were siblings. The retinal degenerative changes and neurological involvement are the most frequently reported (Table 2). It is worth mentioning that there was a striking phenotypic heterogeneity between the two described siblings, although they both tested negative for *NPHP1* homozygous deletion. The two siblings shared NPHP and retinal dystrophy; nevertheless, the younger brother lacked MTS, OMA, and ataxia described in his elder sibling.

In this subset of study patients categorized as JSRD, only one had homozygous deletion of *NPHP1* gene. This patient had both neurological and ophthalmologic involvement as extra-renal manifestations. Notably, he had a more severe form of neurological involvement as compared with the other three JSRD patients who had no *NPHP1* mutational defect. His mental capabilities were profoundly compromised with seizures and his ataxic manifestations were more pronounced as compared with the non-*NPHP1* JSRD patients.

Marked intra-familial variability of associated extra-renal symptoms was observed by Caridi et al in familial cases of JSRDs with homozygous deletion of *NPHP1* (nephrocystin).\(^40\) They reported neurological defects varying from subtle involvement of cerebellum with thickened peduncle to JSRD, showing the typical neurological signs of JBTS (hypotonia, ataxia, psychomotor delay, and oculomotor apraxia-type Cogan).

In contrast, Parisi et al reported that the subset of JBTS patients with an *NPHP1* deletion have a form of JBTS at the mild end of the clinical spectrum for this disorder, as they were not severely mentally retarded and lacked the respiratory disturbance often seen in this disorder.\(^41\)
The fourth patient described in this work as JSRD had more extra-renal manifestations than the other three JSRD children. She had the typical neurological signs with the distinct MTS, retinitis pigmentosa, congenital hepatic fibrosis, as well as ventricular septal defect that was surgically closed at the age of seven years. No NPHP1 mutation was detected in this patient.

Homozygous NPHP1 deletions, the most frequent NPHP1 mutation known, will be detected with the multiplex approach used in the present study. However, in more than 6% of all NPHP1 cases, the underlying mutation has been found to be a heterozygous deletion combined with a single point mutation. As for the patients with no homozygous NPHP1 deletion, we will investigate for heterozygous deletions in a future study by applying the ligation-dependent probe amplification (MLPA) technique. Further analysis of all other NPHP loci for potential homozygosity in consanguineous families by total genome search for linkage using Affimmetrix SNP arrays is also considered. Sequencing all exons of NPHP genes located within regions of significant homozygosity is crucial to identify the underlying genetic defect.

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Figure 1.
A lgorithm depicting characteristics of the study patients. NPHP: nephronophthisis, JSRD: Joubert syndrome related disorder, CORS: Cerebello oculo renal syndrome
Figure 2.  
(a) Axial brain MRI scan of case no. 15 showing MTS with elongated superior cerebellar peduncles (arrows); (b) renal sonogram of patient no. 7 showing hyperechogenic kidney with multiple cortico-medullary and cortical cysts.  
MRI: magnetic resonance imaging, MTS: molar tooth sign
Table 1

Summary of clinical characteristics of the studied subjects.

| Characteristics          | No. of subjects | %   |
|--------------------------|-----------------|-----|
| Gender                   |                 |     |
| Female                   | 11              | 55  |
| Male                     | 9               | 45  |
| Consanguineous parents   | 15              | 75  |
| Sibling death*           | 8               | 40  |
| Polyuria and polydipsia  | 19              | 95  |
| Anemia                   | 20              | 100 |
| Growth retardation       | 20              | 100 |
| End-stage renal disease  | 15              | 75  |
| Hypertension             | 4               | 20  |

* Presumptive diagnosis was nephronophthisis
Table 2

Extra-renal manifestations in four (20%) of the study patients.

| Extra-renal manifestations                      | Patients (n = 20) | %  |
|------------------------------------------------|------------------|----|
| Mental retardation                              | 4                | 20 |
| Dysmorphic facies                               | 3                | 15 |
| Ataxia                                          | 3                | 15 |
| Molar tooth sign                                | 3                | 15 |
| Oculomotor apraxia                              | 3                | 15 |
| Retinal dystrophy/retinitis pigmentosa          | 4                | 20 |
| Congenital hepatic fibrosis                     | 1                | 5  |
| Congenital heart disease*                       | 1                | 5  |

Prominent forehead, broad nasal bridge and hypertelorism

* Ventricular septal defect
Table 3

Clinical and genetic characteristics of the study patients.

| Serial # | Family # | Gender | Age (mo) | Bone age (mo) | Age at onset (mo) | Initial presentation | Extra-renal manifestations | CKD stage at diagnosis | Age at developing ESRD (mo) (RRT) | Cysts on ultrasound | Histopathologic triad | NPHP1 homozygous deletion |
|----------|----------|--------|----------|---------------|------------------|----------------------|--------------------------|-------------------------|--------------------------------|-----------------|-----------------|--------------------------|
| 1        | A1421 II1| F      | 168      | 108           | 36               | P&P, Anemia, GR     | OMA, RP, MR, MTS, CHF, VSD | 5 168                   | [RTx] b             | +               | ND b           | No                       |
| 2        | A1451 II4| F      | 48       | 15            | 24               | P&P, Anemia, GR     | –                       | 4                       | 60 [HD]              | +               | +              | No                       |
| 3        | A1937 II4| F      | 108      | 68            | 48               | P&P, Anemia, GR     | Anemia, GR, episode of generalized edema | –                       | 5                   | 108 [HD]       | +              | No                       |
| 4        | A1937 II6| F      | 48       | 30            | 36               | –                   | –                       | 2                       | –                   | 0               | ND d           | No                       |
| 5        | A1944 II2| M      | 9        | 2             | 5                | FTT, Dehydration P&P, Anemia | –                       | 5                       | 9 [PD]              | +               | ND d           | No                       |
| 6        | A1967 II6| F      | 144      | 106           | 108              | P&P, Anemia, GR     | –                       | 5                       | 144 [HD]            | +               | +              | No                       |
| 7        | A2202 II1| M      | 42       | 30            | 12               | P&P, Anemia, GR     | –                       | 5                       | 32 [HD]             | +               | +              | Homozygous deletion |
| 8        | A2228 II3| F      | 132      | 86            | 48               | P&P, Anemia, GR     | –                       | 4                       | –                   | +               | ND d           | Homozygous deletion |
| 9        | A2229 II5| F      | 78       | 45            | 36               | P&P, Anemia, GR     | –                       | 5                       | 84 [HD]             | 0               | ND d           | No                       |
| 10       | A2245 II4| F      | 89       | 63            | 50               | P&P, Anemia, GR     | –                       | 5                       | 89 [HD]             | +               | ND d           | No                       |
| 11       | A2324 II3| M      | 84       | 54            | 36               | P&P, Anemia, GR     | MR, RD OMA, RD, MR, MTS | 3                      | –                   | 0               | +              | No                       |
| 12       | A2324 II1| M      | 120      | 84            | 36               | P&P, Anemia, GR     | –                       | 5                      | 84 [HD]             | +               | ND d           | No                       |
| 13       | A2325 II1| M      | 144      | 96            | 84               | P&P, Anemia, GR     | –                       | 5                      | 144 [HD]            | +               | ND d           | Homozygous deletion |
| 14       | A2325 II2| M      | 120      | 72            | 48               | P&P, Anemia, GR     | –                       | 4                      | 130 [RTx]           | 0               | +              | Homozygous deletion |
| Serial # | Family # | Gender | Age (mo) | Bone age (mo) | Age at onset (mo) | Initial presentation | Extra-renal manifestations | CKD stage at diagnosis | Age at developing ESRD (mo) (RRT) | Cysts on ultrasound | Histopathologic triad | NPHP I homozygous deletion |
|---------|----------|--------|----------|---------------|------------------|----------------------|-------------------------|------------------------|--------------------------|-----------------|---------------------|--------------------------|
| 15      | A2371 III | M      | 108      | 66            | 48               | P&P, Anemia, GR     | Seizures, OMA, RP, MR, MTS | 5                      | 108 [HD]                 | 0               | ND                  | Homozygous deletion   |
| 16      | A2413 II6 | F      | 126      | ND            | 48               | P&P, Anemia, GR     | -                      | 5                      | 126 [HD]                 | +               | ND                  | Homozygous deletion   |
| 17      | A2412 II1 | F      | 125      | ND            | 50               | P&P, Anemia, GR     | -                      | 5                      | 97 [HD]                  | 0               | ND                  | No                       |
| 18      | A3059 II3 | F      | 97       | 59            | 48               | P&P, Anemia, GR     | -                      | 5                      | 97 [HD]                  | 0               | ND                  | No                       |
| 19      | A3060 II8 | M      | 119      | ND            | 60               | P&P, Anemia, GR     | -                      | 5                      | 107 [HD]                 | +               | ND                  | No                       |
| 20      | A3061 III | M      | 5        | ND            | 3                | P&P, Anemia, FTT, Dehydration | -                      | 5                      | 5 [PD]                   | +               | +                   | No                       |

*RRT: renal replacement therapy, RTx: renal transplantation, HD: hemodialysis, PD: peritoneal dialysis, ND: not done. Histopathological triad: tubular basement membrane thickening and disruption, interstitial infiltration and fibrosis, and tubular atrophy and dilatation with or without cyst formation. P&P: polyuria and polydipsia, FTT: failure to thrive, GR: growth retardation, RP: retinitis pigmentosa, RD: retinal dystrophy, OMA: ocular-motor apraxia, MR: mental retardation, MTS: molar tooth sign, CHF: congenital hepatic fibrosis, VSD: ventricular septal defect, ESRD: end-stage renal disease*