Molecular Genetic Studies of Tunisian Patients with Juvenile Nephronophtisis

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Abstract: Juvenile nephronophtisis (NPHP), a rare recessive cystic kidney disease, represents the most common genetic cause of end-stage renal disease in the first two decades of life. A few studies have investigated the clinical and molecular characteristics of NPHP and related ciliopathies in the Tunisian population. We reported the results of the largest molecular genetic investigation. Since 2004, 71 patients were recruited, 33 children and 38 adults, with a median age of 11 and 32.7±6.1 years and belongs to 27 and 34 unrelated families respectively. Mutations causing NPHP were detected in 10 index cases (14%), belongs to nine independent families. Eight of them (7 Children, one adult) were homozygous for the NPHP1 gene deletion. For the two others, we detected two NPHP4 mutations in homozygous state: a novel missense mutation His1363Pro and a deletion of exons 2 and 3. After relatives investigations the final number increases to 29 patients, 18 carrying NPHP1 deletion and 11 with NPHP4 mutations. Extra-renal abnormalities were present in 27.5% of patients, including retinitis pigmentosa, neurological defects or hearing loss. This detection of the NPHP variations have important implications for genetic counseling towards the identification and care of asymptomatic siblings, then planning of renal replacement therapy.

Keywords: Nephronophtisis, NPHP1 deletion, NPHP4 mutations, Tunisian patients

1. Background

Nephronophtisis (NPH), an autosomal-recessive cystic kidney disorder, is a major genetic cause of chronic renal disease that occurs during childhood and adolescence [1, 2]. According to age at onset of end-stage renal disease (ESRD), three forms of NPH have been described: infantile, juvenile (the most frequent) and adolescent [3].

In the juvenile form, initial symptoms are relatively mild, usually start at 4 to 6 years of age, and consist of polyuric polydipsia, secondary enuresis, and anemia followed by progressive renal failure, resulting in ESRD around 13 years of age [4]. The main histological findings are tubular atrophy with irregularly thickened tubular basement membranes, in association with interstitial fibrosis and cysts located at the corticomedullary junction and medulla [3]. Extra-renal manifestations may be present and include mainly retinal impairment of variable severity. The clinical and histological presentation is similar in the adolescent form, with the exception of delayed occurrence of ESRD (median age: 19 years). Clinical and histological features of the infantile form differ sharply from the two others: ESRD usually occurs in the first 2 years of life, and patients present enlarged kidneys with widespread cyst development [3, 5].

To date, mutations in 13 genes (NPHP1-NPHP11, AHI1, and CC2D2A) have been demonstrated to give rise to NPH when mutated. [6-19]. The corresponding proteins, have various subcellular localizations, including the primary cilia, focal adhesion and adherents’ junction and suggesting a crucial role in the integrity and architecture of renal tubular epithelial cells [3]. The most frequent genes responsible of juvenile NPH are NPHP1 and NPHP4 [3]. Homozygous deletions in the NPHP1 gene, spanned 290 kb in the locus 2q13, is the most frequently found genetic abnormality (70% of patient) and account for approximately 21% of all NPH cases, whereas the other genes contribute less than 3% each. Heterozygous deletion is found in 6% of patients, with concomitant point mutation of the NPHP1 gene on the second allele. [20]

The NPHP4 gene, located on chromosome 1p36 encodes a 1,426 amino acid protein called nephrocystin-4/nephroretinin [10]. This highly conserved protein interacts with nephrocystin-1 (NPHP1 product) and involved in the same intracellular signalling pathway [21]. NPHP4 mutations can cause isolated NPH, NPH with retinitis pigmentosa (RP) or NPH with oculomotor apraxia [20].

The aim of this study was to characterise the different variations causing juvenile NPH as well as the clinical variation associated to these alterations in patients originating from central and southern of Tunisia.

2. Patients And Methods

2.1 Patients

Between 2004 and 2012, 71 patients (children and adults) belongs to 61 unrelated families were referred for CRF to hemodialysis unity of pediatric department and Nephrology
Diagnosis of NPHP was based on clinical manifestations (polyuric-polydipsia, growth retardation), biological findings (hypernatremia, urinary hypo-osmolality) and kidney ultrasound (normal or small-sized kidneys with hyperechogenicity and/or medullary cysts) and family history. All patients had molecular genetic diagnosis without renal biopsy and histological characterisation of NPHP. In addition, they underwent routine clinical tests every 6 months, including the evaluation of renal and hepatic function, kidney/liver ultrasonography, and eye examination. Segregation analysis and clinical assessment in all available family members was made systematically after mutation detection in the index case. Asymptomatic siblings that carried mutation underwent urine concentration tests every 6 months.

2.2 NPHP1 Deletion Screening

Patients with suspected diagnosis of NPH were screened for the presence of homozygous NPHP1 deletions. Genomic DNA was isolated from peripheral blood leukocytes as described previously [22]. As a first step, all 61 families were tested for homozygous deletion of the NPHP1 region using the PCR primers 765F2L, 804/6 and 187.41 [2]. Patients without homozygous NPHP1 deletion were tested for heterozygous deletion by quantitative multiplex PCR using primers in NPHP1 exons 14 and 15 as well as in exon 4 of NPHS2 as a reporter [23].

2.3 Microsatellite Homozygosity Mapping

Pediatric patients with neither homozygous nor heterozygous NPHP1 deletions, underwent microsatellite homozygosity mapping for NPHP1, NPHP3, NPHP4, NPHP5 and NPHP6 loci (Table I). In families potentially linked to one of these NPH genes, direct sequencing of the coding exons and the adjacent intronic junctions was performed. PCR products of the microsatellite markers were separated on a 3% agarose gel, stained with ethidium bromide and visualized by ultraviolet transillumination. Gels were scanned with a molecular imager (BioRad). The segregation analysis of the microsatellite markers was performed with the help of Genetic Data Analysis software (GDA) [24]. All available family members were tested systematically after mutation detection in the index case. The two uncles reached ESRD at the ages of 27 and 30 years, while the brother (patient D-3; 25 year-old) attains the final stage in 20 years-old and received a living related donor kidney transplant from his mother. Histological examination of the native kidney removed at the time of transplantation showed extensive interstitial fibrosis, tubular atrophy, and glomerular sclerosis, tubular basement membranes were considerably thickened with multiple cysts of variable size. Currently, no recurrence of the disease is observed, in this patient, and urinalysis is unremarkable. No extra renal manifestation was approved by our institutional clinical research ethics committee.

3. Results

The molecular genetics diagnosis of NPHP revealed three different mutations causing disease, detected in 10 of 71 patients (14%) belongs to nine of the 61 unrelated families (Table II). Families’ investigations revealed 18 others cases. The homozygous deletions of NPHP1 was tested as first diagnosed line for all patients, but mutation screening was not done for all NPHP genes. In fact, only pediatric cases were tested for other suspected genes. Linkage compatibility to the NPHP1, NPHP4 or NPHP3 loci, was found in two, four and one family respectively. The others families were not linked to any of the loci tested. Sequencing analysis detected two NPHP4 mutations. No mutation causing disease was detected in the others sequenced genes.

3.1 NPHP1 Deletion

Homozygous deletions of NPHP1 were detected firstly in 8 index cases (7/33 children (21.2%) and one adult 1/38 (2.6%)); belongs to seven of the 61 families (11.46%). During family investigations, we have detects 10 other cases (9 homozygous and one heterozygous), who increased the number of affected patients to 18 (13 children and 5 adults), with a median age of 16.5 years (ranged 2-34 years).

3.1.1 Pediatric Cases

Seven of the 13 children, (38.8%) were index cases, with a median age of 10.8 years (ranged 7-15 years), and suffering of chronic renal failure at presentation. The six other kids with a median age of 6 years (ranged 2-8 years) were discovered during families’ investigations and belongs to four families FA (3), FB (1), FE (1) and FF (1). All of them were in asymptomatic stage at the time of molecular diagnosis. Five of them have the homozygous deletions and one girl carried the heterozygote deletion of NPHP1. In the last follow-up, signs of CRF were detected in the patient FA/2 (8 years), his level of urinary concentration was 178 mmol/L.

3.1.2 Adult’s Cases

In the present study we report five adult cases with homozygote NPHP1 deletion with a median age of ESRD of 27.1±5.15 years (ranged 20-34 years). Four of them were diagnosed during family investigations. The brother in family C (26 year-old men), discovered his CRF and a homozygous NPHP1 deletion during the study. Whereas all adult patients of the family D, carrying the homozygous NPHP1 deletion, were already in ESRD when the diagnosis was made (figure 1). In fact, the two uncles reached ESRD at the ages of 27 and 30 years, while the brother (patient D-3; 25 year-old) attains the final stage in 20 years-old and received a living related donor kidney transplant from his mother. Histological examination of the native kidney removed at the time of transplantation showed extensive interstitial fibrosis, tubular atrophy, and glomerular sclerosis, tubular basement membranes were considerably thickened with multiple cysts of variable size. Currently, no recurrence of the disease is observed, in this patient, and urinalysis is unremarkable. No extra renal manifestation was...
noted in all Adult patients. The diagnosis of the patient FI-7 was made in occurrence of CRF in absence of extra-renal manifestations.

3.1.3 Clinical Manifestations of Patients with Homozygous Nphp1 Deletion

Clinically, the diagnosis of NPHP1 deletion was made in the absence of polyurio-polydipsy, hematuria and proteinuria. Among the pediatric patients, growth retardation was noted in five ones. And most of them had isolated kidney involvement. Extra-renal manifestations were noted in four patients (4/18, 22.2%). As especially, RP with congenital deafness were observed in two patients (FB-1 and FD-1) and were associated to a moderate cerebral vermis hypoplasia and mental retardation in patient FD-1 (figure 2, 3). Facial dysmorphia was observed in two patients (FF-1, FB-2). It was associated to a mental retardation in patient (FF-1); and complicated in patient FB-2 with ocular anomalies as low vision, nystagmus, hypertelorism, convergent strabismus, low-set ears and cerebral vermis aplasia as “molar tooth sign”. The consanguinity was present in five families and history of renal disease was present in only in family D.

3.2 NPHP4 Mutations

Following homozygosity mapping at the NPHP4 loci, 5 patients with a median age of 10.5 years (ranged 8-13 years) belonging to 4 families were selected for mutational analysis of all 30 exons. We identified two mutations causing disease in two families. A novel homozygous missense mutation His1363Pro in exon 29, with a PolyPhene score of 2.679, was identified in patient from family FH and a complete deletion of exons 2 and 3, was identified in a girl (V-12) from family FI. Family investigation discovered 9 other cases carrying mutations. In the two other families, only an Ala544Gly polymorphism was identified.

The novel missense mutation His1363Pro, was detected in family FH with a familial history of renal disease (Figure 4). This point mutation was not seen in 100 healthy controls. The median age of the four detected patients was 22 years (ranged 11-28 years) at diagnosis and all of them were in ESRD at diagnosis. The p.H1363P mutation caused a mildest disease phenotype resulting mostly in isolated kidney disease with polyuria and polydipsia but without extra-renal anomalies.

Conversely, the homozygote deletion of exons 2 and 3, found in patient FI-1, incite several extra-renal manifestations as mental retardation, hearing loss and RP. The patient belongs to a highly inbred family with a history of renal disease. Our investigation detected 6/14 of her relatives (three brothers and three cousins) bearing the same deletion. All of patients presented a polyuria and polydipsia. RP was detected in 4 of them (Figure 5).

4. Discussion

Juvenile NPH is an uncommon condition that affects girls and boys equally. The disorder has been reported worldwide [24]. The incidence has been reported to be approximately 0.13 for 10,000 live births in Finland, 1 per 50,000 live births in Canada, and 9 per 8.3 million in United States [25, 26]. In Europe, it accounts for 6 to 10% of childhood renal failure. Some cases have been described in Japan, South America, Median-East countries, and very rarely in Africa [27, 28]. To the best of our knowledge no data on the prevalence of the disease is available in North African or Tunisian population.

In our study, juvenile NPH accounted for 9.6% of the chronic and end-stage renal diseases in children and 5.8% in adults referred to our centre between 2004 and 2012. The molecular genetics diagnosis of NPH revealed three different mutations causing disease. We reported 29 detected patients with juvenile NPH, in which 62% carried homozygous NPHP1 deletion and 37.9% carried NPHP4 mutations. Our data confirm the others reports who suggested that large homozygous deletion encompassing the entire NPHP1 gene is the most detected mutation in NPH patients [29,30].

Because of the rarity of the disease and the paucity of specific clinical symptoms, diagnosis of NPH may be very difficult, and is generally made in advanced stage of the disease. Chronic renal failure was present in all our index cases when the diagnosis was made. All of pediatric cases, were discovered in pre-terminal stage, with similar prevalence’s and age of onset of the disease (33%, 11 years) to that reported by other groups (29.4%, 11-13 years) respectively [28,31, 32]. Diagnosis of type I NPHP in adult patients is very uncommon. Usually, ESRD occurs during childhood or adolescence, but exceptionally in adulthood [33-35]. In the present study we report five adult cases with homozygote NPHP1 deletion with a median age of ESRD of 27 years, with a mild form of disease in absence of extra-renal alterations.

Moreover, we reported a novel missense NPHP4 mutation, p.H1363P, detected in homozygous state in four members of family H. All of them displayed renal manifestations only. Clinically, some features are characteristic and point to a diagnosis of NPH such as polyuria-polydipsia. In our study it led to the identification of a NPHP1 mutation in only one index case. Through segregation analysis and clinical assessment in all available family members, polyuria-polydipsia was present in 14.3% of cases carrying NPHP1 deletion and 100% of patients carrying NPHP4 mutations. Proteinuria and hematuria were absent in all cases. Anaemia, usually described in NPHP, was present in 83% of cases. It prompted diagnosis in one patient but it was related to progressive renal insufficiency in the other cases. Only 25% of patients had growth retardation. Hypertension was observed during the evolution of ESRD in one case. Rapid progression to ESRD was observed in most cases (median 1.3 years after presentation), and initiation of dialysis was required between 10 and 26 years of age (median 14.8 years).

Remarkably, intra familial variation was observed, in fact, families with members having identical genotype but different disease manifestations have been detected in four of our families. In fact, clinical features in index cases of families FB, D, F and I, were more complicated than other siblings carrying the same alteration. This suggested that the
age of onset and symptoms of the disease seems to be variable and can be influenced by others factors as consanguinity. In fact, patients who belong to very consanguineous families, present the most complicated phenotype (figures 1, 4, 5).

Extra-renal anomalies have been described in approximately 10–15% of patients with juvenile NPH [20]. The most frequent was retinal dystrophy, which can be either severe, or moderate with mild visual impairment and RP. In our cohort of juvenile NPH, 27.5% (8/29) of cases displayed extra-renal anomalies. These manifestations, such as ocular, neurological disease or deafness were detected in equally 13.8% of patients with homozygous NPHP1 deletions and homozygote deletion of exons 2 and 3 of NPHP4. Senior-Løken syndrome with RP was detected in 20.7% (6/29) of patients. Mental retardation was detected in three cases (10.3%); it was associated to hypoplasia of the vermis and “molar tooth signs” in patients FD-1 and FB-2, respectively, and facial malformation in case FF-1. Neurological symptoms can be part of the clinical spectrum of the homozygous NPHP1 deletion. In fact, similar cases were described by Caridi et al [36] as a ‘milder’ form of Joubert syndrome, with moderate cerebellar vermis hypoplasia and elongation, but not thickening of the superior cerebellar peduncles that seems to be a peculiar aspect of the association. In addition Tory et al. described similar features in patients that had both a homozygous NPHP1 deletion and a heterozygous truncating mutation of NPHP6 [23].

Deafness is a very rare feature reported in NPH. It was detected in 10.3% of our patients. It was congenital in family B, it is likely that it is due to a mutation in other gene(s), especially because other members of this family had deafness without renal disease. In cases of families C and I with NPHP1 deletion and NPHP4 mutation, respectively, deafness is acquired and it can be a part of related neurological disorders.

Kidney biopsies were not performed in our cohort of patients because of small kidney size and diagnostic delays; however diagnosis was firmly established through molecular genetic testing.

NPH could not be excluded in 78.7% (21/33) of pediatric patients’ vs 97.3% of adult cases, who were negative for the tested genes. It was reported that in more than 1,000 families with NPH, the causative genes are still unknown in about 70% of cases, indicating that further genes are involved in the pathogenesis of NPHP [20]. As for adult patients with no homozygous NPHP1 deletion, further analysis of all other NPHP loci is recommended.

As no specific treatment for NPH currently exists, except the renal replacement therapy, early diagnosis and mutation identification in kindred’s are very important for making decisions on the clinical monitoring of asymptomatic individuals and for genetic counselling. New therapies may become available in the future, such as vasopressin receptor antagonists that have proved successful in the pcy murine model of NPH [23].

5. Conclusion

Juvenile NPH was present in 32% of our studied pediatric cohort, with mutations causing disease mainly detected in NPHP1 and NPHP4 genes. Our study revealed heterogeneity in the clinical features of juvenile NPH. Molecular diagnosis plays a very useful role in confirming the diagnosis in the presence of non-specific signs and in patients who do not display all the pathognomonic signs of the disease. Consequently, NPH must be considered among the differential diagnosis of any cause of renal failure of unknown origin. By allowing earlier diagnosis and time for counselling molecular genetic testing can improve the treatment for NPH. The major challenge remains to understand the biological function of nephrocystin proteins, the molecular mechanisms that lead to renal failure, and to develop potential treatments that may prevent or reverse these changes.

6. Acknowledgements

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Conflicts of interest: The authors declare no conflict of interest.

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### Table 1: Markers used for microsatellite homozygosity mapping

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### Table 2: Clinical and Biological features of the report cases at time of NPH diagnosis

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**FIGURES**

F female ; M Male; MR. : Mental retardation ; VH : Vermis Hypoplasia ; D : Deafness ; FD : facial dysmorphy; RP: retinal pigmentosa; JS: Jouber Syndrom; HD homozygous deletion; Hd hemodialysis; OC: Ocular alterations.
Figure 1: Pedigree analysis in family D, presence of homozygote deletion of NPHP1 gene with a notion of deafness, RP, neurological defects in patient IV-5. The cases III-3, III-4 and IV-2 developed ESRD in adulthood.

Figure 2: Pedigree analysis in family B, presence of homozygote deletion of NPHP1 gene with extra renal anomalies. A notion of deafness, RP, mental retardation were detected in patient FB-1 (IV-2). Facial dismorphia, ocular anomalies and neurological defects (JS) were present in patient FB-2 (IV-7).
Figure 3: MRI of patients $FD-1$ (A, B) and $FB-2$ (C). A: MRI axial sections at the pontomesencephalic level. B: Paramedian sagittal sections showing cerebellar vermis hypoplasia in patient $FD-1$. C MRI of patients $FB-2$: Paramedian sagittal sections showing a “molar tooth sign” characteristic of Jobert syndrome.

Figure 4: Pedigree analysis in family $H$ microsatellite homozygosity mapping for $NPHP4$ gene, and presence of the mutation His1363Pro in homozygous state in exon 29.

Figure 5: Pedigree analysis in family $I$, presence of homozygote deletion of exons 2 and 3 in $PHP4$ gene. RP was present in 4 patients. The index case V-12 have RP, mental retardation and deafness.