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QMPSF is sensitive and specific in the detection of *NPHP1* heterozygous deletions

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Abstract

Background: Nephronophthisis, an autosomal recessive nephropathy, is responsible for 10% of childhood chronic renal failure. The deletion of its major gene, *NPHP1*, with a minor allele frequency of 0.24% in the general population, is the most common mutation leading to a monogenic form of childhood chronic renal failure. It is challenging to detect it in the heterozygous state. We aimed to evaluate the sensitivity and the specificity of the quantitative multiplex PCR of short fluorescent fragments (QMPSF) in its detection.
Methods: After setting up the protocol of QMPSF, we validated it on 39 individuals diagnosed by multiplex ligation-dependent probe amplification (MLPA) with normal *NPHP1* copy number (n=17), with heterozygous deletion (n=13, seven parents and six patients), or with homozygous deletion (n=9). To assess the rate of the deletions that arise from independent events, deleted alleles were haplotyped.
Results: The results of QMPSF and MLPA correlated perfectly in the identification of 76 heterozygously deleted and 56 homozygously deleted exons. The inter-experimental variability of the dosage quotient obtained by QMPSF was low: control, 1.05 (median; range, 0.86–1.33, n = 102 exons); heterozygous deletion, 0.51 (0.42–0.67, n = 76 exons); homozygous deletion, 0 (0–0, n = 56 exons). All patients harboring a heterozygous deletion were found to carry a hemizygous mutation. At least 15 out of 18 deletions appeared on different haplotypes and one deletion appeared de novo.
Conclusions: The cost- and time-effective QMPSF has a 100% sensitivity and specificity in the detection of *NPHP1* deletion. The potential de novo appearance of *NPHP1* deletions makes its segregation analysis highly recommended in clinical practice.

Keywords: copy number variation; deletion; nephronophthisis; *NPHP1*; quantitative multiplex PCR of short fluorescent fragments (QMPSF).

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Introduction

Nephronophthisis is an autosomal recessive kidney disorder leading to interstitial fibrosis and tubular basement membrane alterations. It is a common cause of childhood end-stage renal disease, responsible for 10% of the cases. Its classical symptoms, polyuria and polydipsia, are typically not severe enough for the parents to seek medical help. Therefore, when no extra-renal involvement is associated, it typically remains unrecognized till the onset of end-stage renal disease [1]. Genetically, it is highly heterogeneous with 20 genes identified thus far [2]. Among them, *NPHP1* (OMIM #256100) is the most frequently mutated due to a recurrent deletion involving the entire gene [3]. This deletion results from an unequal recombination between two directly oriented low-copy repeats, the size of which (~45 kb) has not yet allowed the identification of the breakpoint [4]. This mechanism makes the *NPHP1* deletion the most common mutation in monogenic forms of childhood chronic renal failure with an allele frequency of 0.24% in the general population [5].

In the majority of the patients, the *NPHP1* deletion is present in the homozygous state making its detection by PCR easily feasible [6]. Nevertheless, when trans-associated to a point mutation or in carrier parents and siblings, its detection in the heterozygous state may be challenging. As the localization of the breakpoint is unknown, a breakpoint-spanning PCR is not feasible. Therefore, in clinical practice and even in medical reports, the detection of the heterozygous deletion is either neglected or necessitates labor-intensive and expensive methods such as comparative genomic hybridization, fluorescent in situ hybridization or multiplex ligation-dependent probe amplification (MLPA) [7–9].

The time- and cost-effective method of quantitative multiplex PCR of short fluorescent fragments (QMPSF) has been widely used in the identification of copy number variations (CNV) [10–18]. We adapted it for the *NPHP1* deletion screening, and here we show its perfect sensitivity and specificity, and the importance of the screening in clinical practice.

Materials and methods

Patients

Forty individuals from 28 families (31 patients and 9 parents), diagnosed between 2010 and 2015 at the 1st Department of Pediatrics, Semmelweis University Budapest with potential *NPHP1*-associated nephronophthisis – i.e. juvenile nephronophthisis, either isolated or with mild Joubert syndrome or late-onset retinopathy [19] – were investigated. Two families were multiplex with two and three affected children. Two families were consanguineous. All families gave informed consent, and the study was approved by the Local Research Ethic Committee (TUKEB 6569-0/2010-1018EKU).

DNA extraction

DNA was extracted based on proteinase K digestion followed by high-sodium chloride treatment to precipitate proteins. DNA concentration was measured by NanoDrop™ 1000 Spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA) and was diluted with TE buffer (10 mM Tris-HCl pH 8.2, 1 mM EDTA) to achieve a final concentration of 10 ng/μL for QMPSF and 20 ng/μL for MLPA.

Multiplex ligation-dependent probe amplification assay

All samples were analyzed by the *NPHP1*-specific reagent kit, covering all 20 exons (SALSA MLPA P387 *NPHP1* probemix; MRC-Holland, Amsterdam, The Netherlands), according to the manufacturer's instructions, with 100 ng genomic DNA. Fragment analysis was performed on ABI 3500 Genetic Analyzer (Applied Biosystems, Foster City, CA, USA), and the results were evaluated using Peak Scanner Software (Thermo Fischer Scientific) and Coffalyser (MRC-Holland).

Validation of the identified deletions

All *NPHP1* deletions – identified by MLPA – were confirmed by a second method in the affected individuals. Homozygous deletions were validated by PCR as previously described [6]. Patients with heterozygous deletion were screened for point mutations in the coding regions and the splicing junctions of *NPHP1* (NM_000272) by Sanger sequencing [4]. The hemizygous state of the point mutations was confirmed by haplotype and segregation analysis. A partial 3′ deletion was confirmed by array CGH.

Quantitative multiplex PCR of short fluorescent fragments (QMPSF)

All samples were investigated by QMPSF. Short, 120- to 321-bp-long fragments of six *NPHP1* (2q13) exons [3, 6, 9, 12, 15, 19] together with two control regions of *HSD17B3* (9q22.32) and *USH2A* (1q41) were amplified by multiplex PCR (Table 1, Figure 1).

The conditions were set up in a patient with a heterozygous deletion and in three control subjects, two of whom was diagnosed with an *HNF1B* deletion and one with amyloidosis, who was also confirmed by MLPA to have a normal *NPHP1* copy number. The multiplex PCR was performed in 30 μL of reaction master mixture containing 0.2 mmol/L of each dNTP, 1.5 mmol/L $MgCl_2$, 0.6 U Immolase DNA polymerase (ImmoMix™, Bioline Inc.), and 0.1–0.4 μmol/L of each primer as specified in Table 1. Twenty-nanogram genomic DNA (2 μL) was added to yield a final volume of 32 μL. The initial denaturation (95 °C for 12 min) was followed by 26 cycles of amplification (95 °C for 45 s, 60 °C for 45 s, 72 °C for 45 s) and a final extension (72 °C for 10 min). This limited number of cycles stops the amplification in the exponential phase keeping the amount of the PCR product proportional to the original amount of the targeted sequence, thus allowing the quantification of the target sequence relative to the control regions. All forward primers were labeled with 6-FAM fluorophore and universal extensions were added to the 5′ end of both forward and reverse primers [13, 16, 20]. Fragment analysis was carried out in the same way as for MLPA.

Four controls (three negative and one with a known heterozygous deletion identified by MLPA) were run in each assay. Results of QMPSF were analyzed only if the peak height of both *HSD17B3* and *USH2A* control regions were above 2000 relative fluorescence units. To determine the gene dosage for each amplified region, dosage quotient (DQ) was calculated: the ratio of the target peak area and the average of *HSD17B3* and *USH2A* peak areas was determined for each patient and was subsequently divided by the controls' average ratio. This gives a theoretical DQ of 1.0 for two *NPHP1* copies and 0.5 for a heterozygous deletion [10, 12, 21, 22]. Following the criteria of the MLPA protocol, we considered a DQ value between 0.3 and 0.7 to reflect a heterozygous deletion and a DQ value between 0.7 and 1.3 to reflect a normal copy number [23, 24].

Table 1: *NPHP1*-QMPSF primer sequences.

Primer	Primer sequence	Product length, bp	Concentration, µmol/L
NPHP1 3F	CCTGCTCAACGACTGAATGA	136	0.1
NPHP1 3R	TCTATTGCCTGCTTTAACTGGA		
NPHP1 6F	CGGGTGATAGGGAAGCTTTT	225	0.2
NPHP1 6R	TCCACCAACCATCAGGTTTT		
NPHP1 9F	TGCAGAAACATGACTGAAAACA	241	0.4
NPHP1 9R	TGTGTTTTGCCTGTGACAGAA		
NPHP1 12F	AGCCACATGGCAACCTAAAA	200	0.4
NPHP1 12R	CAATGTCCTCAAAGAACACCAA		
NPHP1 15F	ACCTCATGGAGGGATCAACA	321	0.4
NPHP1 15R	GCTACCTCTCAGATGCTTCTATTTG		
NPHP1 19F	TTTCTGTTTGCTCTTTAGAGTTCG	120	0.2
NPHP1 19R	GCAAATATGGAGTTCAGTGTGG		
***HSD17B3* 11F**	AAGGCTGCTCCTGACACACT	186	0.2
***HSD17B3* 11R**	CCTCCATCTTCAGCGGACTA		
***USH2A* 41F**	CCTTTCACCAGAGTCCCAGA	273	0.2
***USH2A* 41R**	CCATGGGCTAAGAGCAGAAG		

Primers of control regions are in bold.

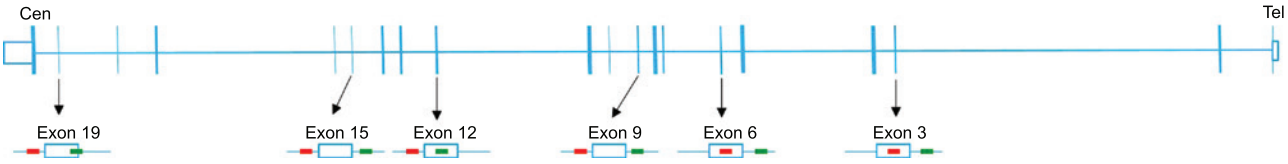

Figure 1: The position of the *NPHP1*-QMPSF primers (2q13, ENST00000393272.3) as shown by green (forward) and red lines (reverse primers). Cen, centrome; Tel, telomere.

Haplotype analysis

The D2S293, D2S340, D2S1893, D2S160, and D2S363 microsatellite markers – located outside the *NPHP1* deletion – were used for haplotype analysis as described previously [25–27].

CGH array

CGH array analysis was performed in a patient who was compound heterozygous for two different deletions. A high-resolution genomic scan using ISCA plus design array of Nimblegen-Roche containing 1.4 M probes per subarray (assembly GRCh37/hg19) was performed in the DNA sample of the patient. This CGX microarray provides a mean average resolution of approximately 15–20 kb. Array CGH analysis was performed according to the manufacturer's protocol. The CGH protocol involves independent labeling of the patient (test DNA) and the reference genomic DNA (Human Genomic DNA, Promega, Madison, WI, USA) with Cy3 and Cy5 dyes using a NimbleGen Dual-Color DNA Labeling Kit (Roche-NimbleGen Inc.). Co-hybridization of these DNAs to a NimbleGen CGH array were performed for 72 h at 42 °C. The subarrays were scanned on NimbleGen MS 200 microarray scanner and data were extracted and analysed using NimbleScan, SignalMap, and Deva 1.1 softwares (Roche NimbleGen Inc.).

Results

High rate of NPHP1 deletion in juvenile nephronophthisis

Out of the 31 patients with juvenile nephronophthisis, we found 16 (52%) patients (14/28 families) to carry *NPHP1* deletion (c.(?_-1)_(*1_?)del) on at least one allele by MLPA (Table 2). Nine unrelated patients were homozygous and five patients from three families were heterozygous for the deletion. The homozygous deletion was confirmed in all cases by PCR and all five patients with a heterozygous deletion were found to carry a hemizygous point mutation, two of which (c.489delT, p.Phe163Leufs*19, and c.656C > A, p.Ser219*) are novel (Figure 2).

Two more unrelated patients were compound heterozygous for the classical whole-gene deletion and a novel 3′ partial deletion (c.(1810 + 1_1811-1)_(*1_?)del) involving the last three [18–20] exons. The latter was confirmed by array CGH in one of the two patients (hg19

Table 2: *NPHP1* mutations of the patients.

Families	Paternal allele	Maternal allele
F10, F12, F15, F63, F67, F70, F84, F97, F158	del[a]	del[a]
F36	c.84_87delTTCT, p.Ser29Argfs*4	del[a]
F38	c.489delT, p.Phe163Leufs*19	del[a]
F249	c.656C>A, p.Ser219*; del[b]	
F106	Exon 18–20 deletion[c]	del[a]
F203	del[a]	Exon 18-20 deletion[c]

[a]del.: c.(?_-1)_(*1_?)del, [b]unknown segregation, [c]c.(1810+1_1811-1)_(*1_?)del or g.(110850451_110862515)_(110888028_110889217)del.

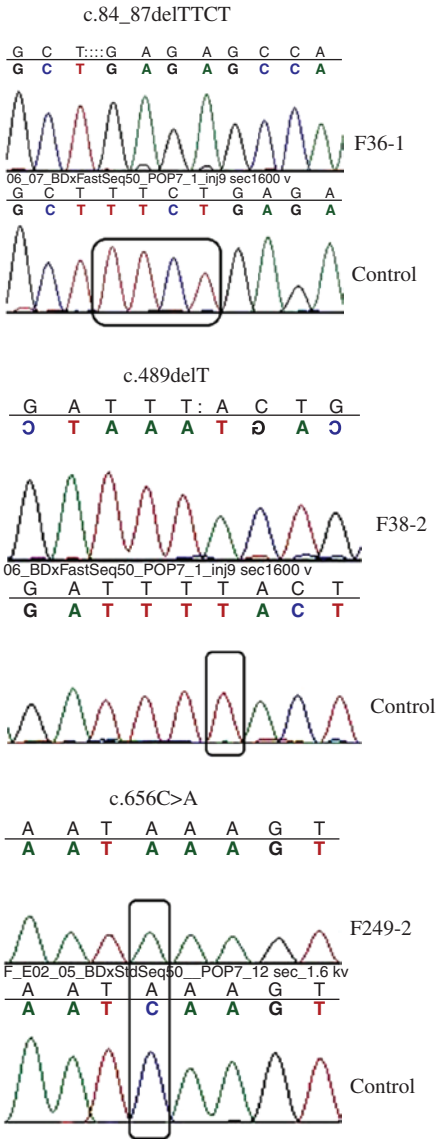

Figure 2: Chromatograms of the hemizygous mutations.

chr2: g.(110850451_110862515)_(110888028_110889217)del, F106-1, Figure 3) and the second patient (F203-2) was shown to carry the same haplotype on the 3′ partially deleted *NPHP1* allele, reflecting a founder effect (Figure 4).

NPHP1 deletions typically result from independent events

Out of the 16 deletions of non-consanguineous families, we found one deletion to appear de novo, on the paternal allele (F84). The biological paternity was confirmed by haplotype analysis (Figure 5).

Furthermore, among the 18 haplotypes with whole gene deletion (taking into account the alleles of the two consanguineous families as a single allele), we found at least 15 different alleles indicating a high rate of independent deletions, which is in accordance with previous results and contrasts the founder 3′ partial deletion (Figure 4) [4, 28].

Intra-experimental variability of *NPHP1*-QMPSF is low

We found the intra-experimental variability of *NPHP1*-QMPSF – through investigating a control subject and a patient with a heterozygous deletion in ten parallel measurements – to be low: $DQ_{control} = 0.98$ (median; quartiles, 0.96–1.01; range, 0.86–1.09); $DQ_{het.del} = 0.51$ (median; quartiles, 0.50–0.53; range, 0.45–0.57).

The specificity and sensitivity of QMPSF are perfect in the detection of an *NPHP1* deletion

There was a clear-cut difference in the DQ values of 17 control subjects, 13 parents and patients with heterozygous deletion, and 9 patients with homozygous deletion in all six exons tested (Figures 6 and 7), as also indicated by the overall values: $DQ_{control} = 1.05$ (median; quartiles, 0.99–1.08; range, 0.86–1.33); $DQ_{het.del} = 0.51$ (median; quartiles, 0.50–0.54; range: 0.42–0.67); $DQ_{hom.del} = 0$ (median; range, 0–0).

The two patients with compound heterozygous deletions were properly found to be heterozygous for exons

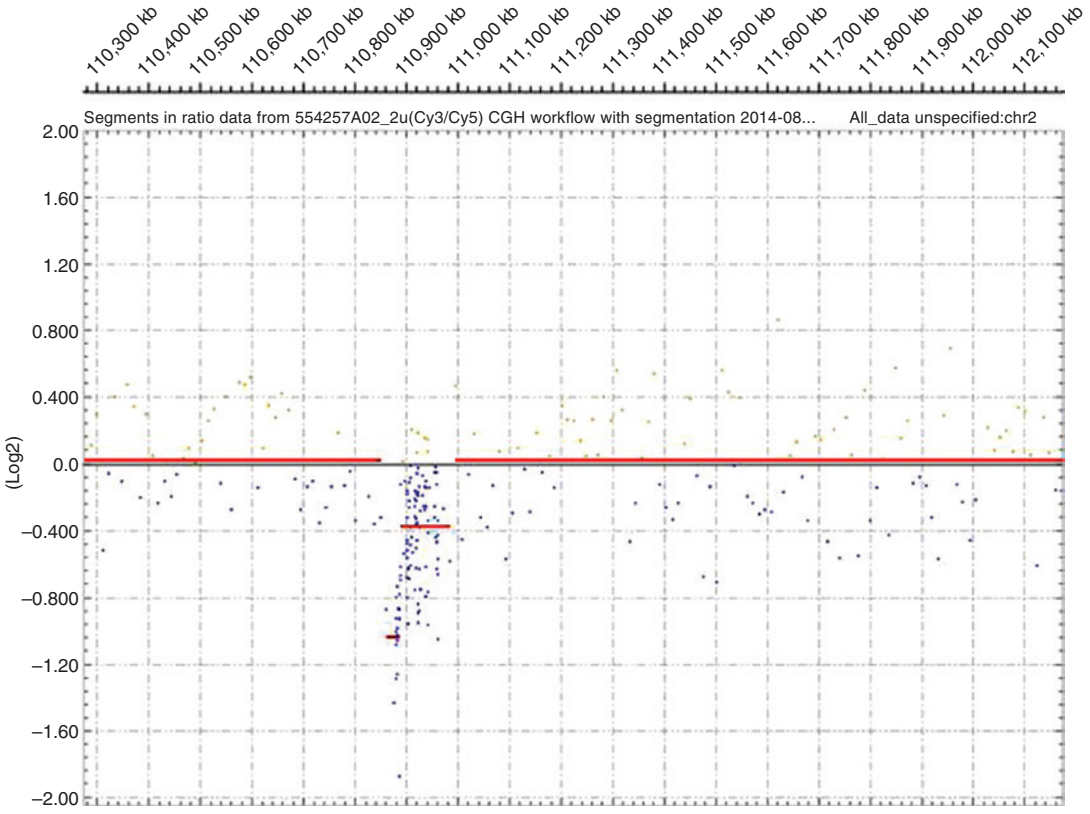

Figure 3: Array CGH of patient F106-1.
Compound heterozygous deletion in the *NPHP1* gene: while both alleles are deleted (score – 1.05) in the region chr2:g.110862515_110888028 (on Assembly GRCh37), a heterozygous deletion (score – 0.38) is found in the region g.110888028_110985511. This reflects the trans-association of the classical whole gene deletion (g.(110850451_110862515)_(110985511_110996482)del) and a partial 3′ deletion (g.(110850451_110862515)_(110888028_110889217)del), and defines the 5′ breakpoint of the partial deletion in intron 17.

Marker	gen. pos. (GRCh37)	Patients																					
		F10-2		F12-2		F15-1		F36-1		F36-2		F67-2′		F70-2		F84-2		F97-2		F106-1		F158-6′		F203-2	
D2S293	107.3	3	6	3	10	2	11	1	2	5	2	4	4	2	9	2	7	3/7	3/7	7	4	6	6	3	4
D2S340	108.9	4	3	3	5	3	6	5	3	4	5	3	3	2	3	5	5	1	5	3	4	3	3	4	4
D2S1893	109.8	1	9	11	6	9	6	9	9	6	10	9	9	9	9	10	10	6	9	10	6	10	10	11	6
NPHP1	110.9	Del	Del	Del	Del	Del	Del	Del	pm	Del	pm	Del	Del	Del	Del	Del	Del	Del	Del	Del	3′del	Del	Del	Del	3′del
D2S160	113.0	5	6	9	6	6	7	4	2	5	5	6	6	2	5	6	5	5	7	1	3	6	6	5	6
D2S363	117.4	4	1	2	2	1/2	1/2	2	5	1/2	1/2	2	2	1	2	2	2	1	1	2	1	4	4	2/3	2/3

Figure 4: Haplotype of patients with *NPHP1* deletion.
Del: whole gene deletion (c.(?_-1)_(*1_?)del), 3′del: exon 18–20 deletion (c.(1810+1_1811-1)_(*1_?)del), pm, point mutation; a, products of consanguineous marriages. Similar haplotypes are highlighted by the same gray shade.

3, 6, 9, 12, and 15 deletions and to be deleted on both alleles in exon 19. Thus, with the exception of a DQ of 1.33 erroneously suggesting a duplication for exon 3 in a patient with a normal copy number (F2-1), results for all exons of all patients correlated perfectly with the MLPA results. Thus, all and only the 76 heterozygously deleted exons were identified by QMPSF to be deleted in the heterozygous state, giving 100% sensitivity and 100% specificity for the identification of a heterozygous deletion.

Discussion

The *NPHP1* gene deletion is one of the most common mutations leading to a severe monogenic disorder: it is only

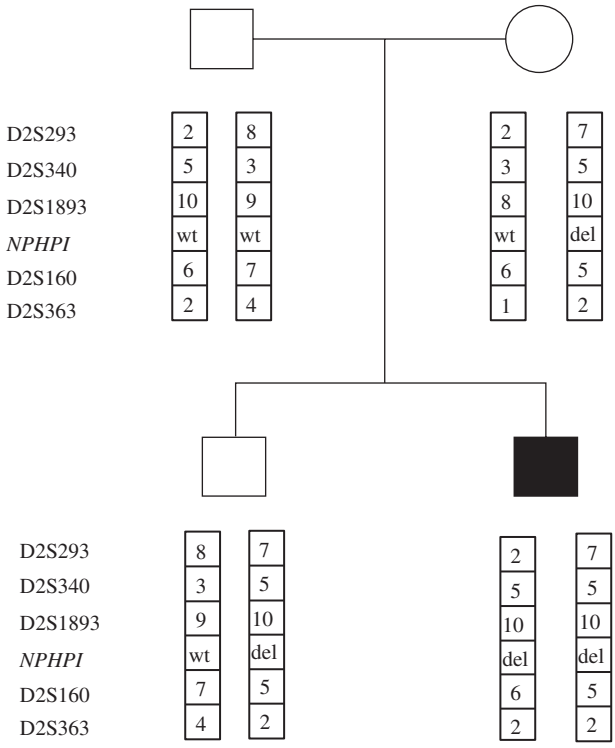

Figure 5: Haplotype analysis of the family F84 with a de novo deletion.
We found no recombination in the *NPHP1* region between the two paternal chromosomes suggesting that the deletion resulted from an intrachromosomal loop formation.

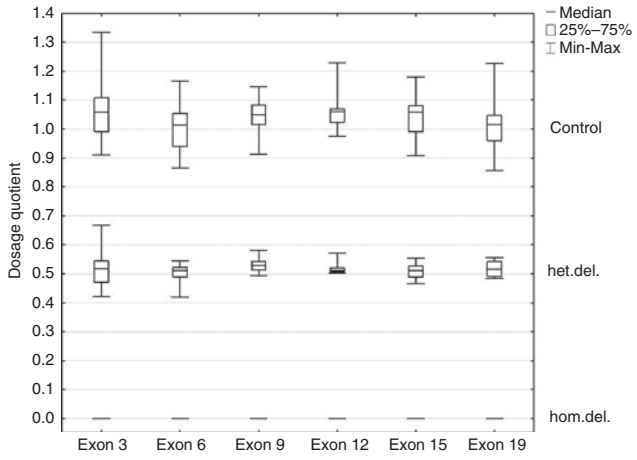

Figure 6: Dosage quotient of *NPHP1* exons determined by QMPSF in patients and control subjects.

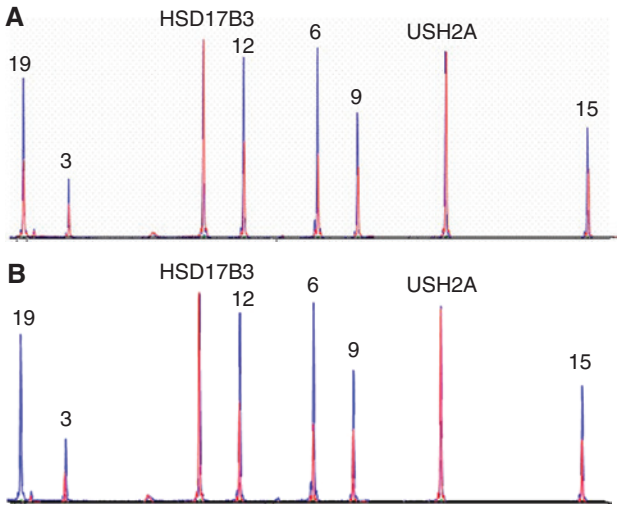

Figure 7: Electropherograms of either a patient with heterozygous deletion (A) or a patient (F106-1) with compound heterozygous deletion (B) (in red), superimposed on the electropherograms of a control subject (in blue).
While control regions (*HSD17B3* and *USH2A*) show a similar peak height in patients and controls, heterozygous deletion reduces the peak height by half and a deletion on both alleles (B, patient F106-1, exon 19) abolishes it.

four times less frequent than the *CFTR* p.Phe508del mutation, and twice as frequent as the *NPHS2* p.R138Q mutation in the European population [5, 9, 29]. As such, to the best of our knowledge, it is the most common mutation leading to chronic renal failure in childhood. In accordance with its frequent causal role in nephronophthisis, we found it in the families with juvenile nephronophthisis in the homozygous and heterozygous state in 32% and 18%, respectively. Such a high *NPHP1* mutation rate was found in other single center cohorts [9, 30, 31], while a lower rate was reported in worldwide cohorts [2], probably due to the less stringent clinical inclusion criteria. Although the majority of the patients carry the *NPHP1* deletion in the homozygous state, we found one third of the patients with *NPHP1* deletions to be compound heterozygous for the whole gene deletion and either a point mutation or a partial deletion. A similar ratio was reported in other cohorts [9, 32], making the identification of *NPHP1* deletion in the heterozygous state important. One could argue that sequencing of *NPHP1* would allow the identification of the trans-associated hemizygous mutations and thus it is useless to identify the *NPHP1* deletion in the heterozygous state. Nevertheless, differentiation of the homozygous and hemizygous state of the trans-associated mutations is possible only if parental DNA samples are available, and even if they are, it is difficult or even impossible to conclude whether the deletion is inherited or appeared de novo. This latter seems to be crucial in case of the *NPHP1* gene deletion, which – in contrast to the vast majority of the recessive mutations or the novel 3′ partial deletion – is not a founder mutation, but arises from independent events [4, 28]. This is very well reflected in our study by the detection of a de novo deletion and by the very different haplotypes of the deleted alleles.

Even among families with homozygous deletion, only the consanguineous families carried the same haplotype on the two alleles. The independent origin of the deletions also explains its similarly high frequency in different geographic area [9, 30, 31]. Two mechanisms have been proposed to explain the appearance of an *NPHP1* deletion: an unequal recombination secondary to (1) chromosome misalignment following an intrachromosomal loop formation or secondary to (2) unequal crossing over between the two homolog chromosomes leading to a deletion on one and a duplication on the other homologue [4]. We found no crossing over and thus suggest the first mechanism to be causal in the family with the de novo deletion (Figure 5). The de novo appearance dramatically reduces the risk of recurrence among the siblings, but does not abolish it as parental mosaicism was found in 4% of families with de novo CNV [33]. As the de novo rate of the *NPHP1* deletion – 1/16 in the present and 1/14 in a previous study [34] – is not negligible, it is crucial to verify the carrier status of the parents. Furthermore, given the carrier frequency of ~1 : 200 in the general population, one could even consider screening the partners of mutation carriers. All these aspects make the detection of the heterozygous *NPHP1* deletion important both for the genetic diagnosis and for genetic counseling. However, the carrier status of the parents has rarely been investigated [35].

Given the frequency of the *NPHP1* deletion and the difficulty of its detection by multiple parallel sequencing, its targeted genetic screening should precede any other genetic test once the clinical diagnosis of juvenile nephronophthisis is raised [1]. As QMPSF is 3.5× less expensive in our setting than MLPA, and replaces the four-step procedure of MLPA by a single one, we tested it for the detection of *NPHP1* deletion. The diagnosis of *NPHP1* deletion was primarily established by MLPA and confirmed by a second test in all patients. Within this cohort, we found both the specificity and the sensitivity of QMPSF to be 100% for the detection of both the heterozygous and the homozygous *NPHP1* deletion. We also properly identified a compound heterozygous deletion by QMPSF, though our method covers only six out of 20 exons. To achieve such a high sensitivity and specificity, it is essential to properly stop the amplification before the saturation and thus to keep the amount of the PCR product proportional to the original amount of the targeted sequence. A potential disadvantage of our QMPSF method is that it misses those CNVs that are located in-between the targeted six exons. Though no such a small CNV has been reported thus far in *NPHP1*, they would go undetected by this method. On the other hand, though duplications of *NPHP1* are not implicated in the pathogenesis of nephronophthisis and thus could not be investigated, they have been reported in patients with autism [36] and are expected to be detectable by QMPSF.

We thus conclude that *NPHP1*-QMPSF is perfectly suitable for the detection of the *NPHP1* deletion even in the heterozygous state and thus suitable for the clinical diagnosis. As the *NPHP1* deletion may occur de novo, the carrier status of the parents is highly recommended to be verified in clinical practice.

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