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ARTICLE



Combining *RSPH9* founder mutation screening and next-generation sequencing analysis is efficient for primary ciliary dyskinesia diagnosis in Saudi patients

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Primary ciliary dyskinesia (PCD) is a clinically and genetically heterogeneous ciliopathy. Dysfunction of motile respiratory and nodal cilia results in sinopulmonary symptoms associated with laterality defects (LD) found in half of the patients. The molecular basis of the disease is insufficiently investigated in patients originating from the Arabian Peninsula. In a group of 16 unrelated Saudi patients clinically suspected of PCD and among whom only 5 (31%) had LD, we first screened by PCR-RFLP two founder mutations, *RSPH9* c.804_806del and *CCDC39* c.2190del previously identified in patients from the Arabian Peninsula and Tunisia, respectively. When negative, targeted panel or whole-exome sequencing was performed. Three patients were homozygous for the mutation in *RSPH9*, which encodes an axonemal protein that is absent from nodal cilia. None of the patients carried the *CCDC39* founder mutation frequent in Tunisia. NGS analysis showed that nine patients had homozygous mutations in PCD genes. In total, sequential RFLP and NGS analysis solved 75% (12/16) of cases and identified ten distinct mutations, among which six are novel, in nine different genes. These results, which highlight the genetic heterogeneity of PCD in Saudi Arabia, show that the *RSPH9* c.804_806del mutation is a prevalent mutation among Saudi patients, whereas the *CCDC39* c.2190del ancestral allele is most likely related to the Berber population. This study shows that *RSPH9* founder mutation first-line screening and NGS analysis is efficient for the genetic exploration of PCD in Saudi patients. The *RSPH9* founder mutation accounts for the low rate of LD among Saudi patients.

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INTRODUCTION

Primary ciliary dyskinesia (PCD) is an inborn ciliopathy caused by dysfunction of motile cilia, which results in chronic sinopulmonary symptoms (SPS) due to impaired mucociliary clearance [1]. Patients show recurrent respiratory infections, and chronic sinusitis and otitis [2]. Neonatal respiratory distress (NRD) is observed in more than 75% of PCD full-term neonates [3]. About half of the patients also show laterality defects (LD) such as *situs inversus* (Kartagener syndrome) or heterotaxy, a consequence of the altered nodal flow that is driven by nodal mono-ciliated cells [4]. The presence of at least one of these signs can lead to suspect PCD [5]. Several diagnostic tests can concur to confirm the diagnosis, although none of them is a gold standard [6]. Low nasal nitric oxide (nNO) levels are evocative of PCD, as well as ultrastructure defects in airway cilia shown by transmission electron microscopy (TEM), or the absence of axonemal components shown by immunofluorescence. High-speed video-

microscopy analysis can also highlight the abnormal ciliary beat frequency and/or pattern [5]. However, these tools are either labor-intensive and/or have limitations [7–9].

PCD is a highly heterogeneous genetic condition as about 50 genes have been implicated so far [10]. Its inheritance pattern is mostly autosomal recessive, but an autosomal dominant inheritance has been also reported in a few PCD patients harboring mutations in *FOXJ1*, a multiciliogenesis factor [11]. In addition, three genes have been implicated in an X-linked phenotype (*RPGR*, *DNAAF6*, and *OFD1*) [12–15]. Genetic testing is now very appealing especially with advances in DNA sequencing technology, although data interpretation can be challenging. This massive sequencing has increased the pace of PCD-gene discovery [16]. In recent years, using different next-generation sequencing (NGS) approaches (whole-exome sequencing (WES) and targeted panels), many families have been explored and the causative genetic defects have been identified in various populations [17–22]. PCD genes encode axonemal proteins,

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Table 1. Clinical data and age at presentation for the 16 patients

Patient ID	Gender	Age at clinical diagnosis	LDs	NRD	Bronchitis	Bronchiectasis	Rhinosinusitis	Otitis	PICADAR score (total/14) ^a
P12	F	6 months	–	–	+	–	+	+	4
P17	M	Birth	–	+	+	–	+	+	6
P20	F	Birth	–	+	+	–	–	+	5
P1	M	Birth	–	–	+	+	+	–	3
P13	M	Birth	SIT	+	+	–	–	–	8
P2	F	1 year	–	+	+	+	+	+	6
P3	M	Birth	–	+	+	–	+	+	6
P6	M	Birth	SIT	+	+	–	–	–	8
P11	F	Birth	SIT	+	+	–	+	+	12
P16	F	Birth	–	+	+	–	–	–	4
P19	F	1 month	SIT	–	+	–	+	+	8
P14	M	Birth	DX	+	+	–	+	+	10
P4	F	6 months	–	–	+	+	+	NA	3
P7	F	13 years	–	NA	+	+	+	–	3
P15	F	Birth	–	+	+	–	+	+	6
P18	F	6 months	–	–	+	–	+	+	4

M male, F female, LD laterality defect, SIT situs inversus totalis, DX isolated dextrocardia, NRD neonatal respiratory distress, NA not available

^aPICADAR (Primary Ciliary Dyskinesia Rule) score predicts if patients are likely to have PCD according to Behan et al. [40]

cytoplasmic proteins involved in axonemal components assembly, and factors driving multiciliogenesis. Most of the mutations (85%) lead to a premature stop codon [23]. About 75% of the mutations are private as they are only identified in a few families, and frequently in a single one [24]. Some of the recurrent mutations have been linked to ethnic origin or geographical location and probably emerged from a founder effect. For instance, *DNAI1* c.48+2dup mutation in Caucasian patients [25], *HYDIN* c.922A>T, p.(Lys308*) mutation in the Faroe Island population [26]. Likewise, recurrent and founder mutations have been reported in Arabic families. The *RSPH9* c.804_806del founder mutation in exon 5 has been previously identified in several Saudi families [27, 28]. Recently, we have reported that *CCDC39* c.2190del in exon 16 is the most recurrent mutation in Tunisian families and results from a founder effect [20]. This mutation, as well as the founder *RSPH9* one, eliminates a recognition site for the *MbolI* restriction enzyme and therefore could be directly and easily screened. In this study, we present direct and rapid screening of those two founder mutations (*RSPH9* c.804_806del and *CCDC39* c.2190del) by PCR-enzyme digestion and Sanger sequencing, before proceeding with targeted panel or WES in a cohort of Saudi PCD patients.

MATERIALS AND METHODS

Patients and subjects

The 16 independent patients included in this study were clinically suspected of PCD and were recruited from 2018 to 2020. Patients were followed in pediatric pulmonology departments of four Saudi cities (Jeddah, Makkah, Taif, and Hail). For each patient, clinical features were collected in a standardized data sheet including information about NRD, LD, chronic wet cough, bronchitis, bronchiectasis, sinusitis, and otitis. Patients included in the study had at least one of the three following phenotypes: SPS, NRD, and LD. Their clinical presentation has been graded with the PICADAR score. Blood samples were obtained from probands and their parents when available. This study was approved by the institutional review board of hospitals. Informed consent was obtained from all subjects or from their guardians.

PCR and restriction-fragment length polymorphism (RFLP)

Genomic DNA was extracted from blood samples using a salting-out method [29]. PCR amplification of candidate exons was performed with

the GoTaq[®] G2 Hot Start Master Mix kit (*Promega #M7422*). PCR reactions were performed at 60 °C annealing temperature with 100 ng of genomic DNA and 0.1 μM of each specific primer (*CCDC39* exon 16: 5'-TGGCTACTACATGCCCATAGT-3' and 5'-GTCTTCTGGTGAAGAGCAA-GAG-3'; *RSPH9* exon 5: 5'-CCAGTGAACCATAGCAGCT-3' and 5'-AACAGG-CAGGCCAAGTTCAC-3'). The c.2190del *CCDC39* founder mutation was screened first in all probands by digestion of exon 16 amplicon with *MbolI* as reported in our previous work [29]. The same enzyme was used to screen the c.804_806del *RSPH9* founder mutation, in probands without LD, by digestion of the exon 5 PCR product [27]. The presence of this pathogenic mutation has been confirmed by Sanger sequencing.

Targeted panel and whole-exome sequencing (WES)

NGS sequencing was performed for probands in which RFLP screening was negative, in Armand-Trousseau Hospital Molecular Genetics Department, France. The targeted panel analysis was based on a custom design encompassing the 48 known PCD genes [10] and about 250 candidate genes (SeqCap EZ Choice, NimbleGen Roche Diagnostics). Exome sequencing was performed through a MedExome capture (Roche diagnostics). Libraries were sequenced on a MiSeq (panel) or a NextSeq (exome) sequencer (Illumina). Data analysis was carried out using two in-house pipelines (based on BWA and Bowtie aligners). Sequencing depth of the regions of interest (coding regions of PCD genes) was over 50× for the targeted panel and 25× for the exome sequencing. Search for copy number variations was carried out by read depth ratio analysis of NGS data [30]. Identified variants were classified following the standards and guidelines of the American College of Medical Genetics [31]. The frequency of variants in the general population was searched in the gnomAD database (<https://gnomad.broadinstitute.org/>) [32]. The effect of the detected variants on splicing was checked using SpliceAI tool (<https://github.com/Illumina/SpliceAI>) [33]. The pathogenicity predicting score CADD was also calculated for each variant (<https://cadd.gs.washington.edu/score>) [34].

Sanger sequencing

The intrafamilial segregation of pathogenic mutations was performed by Sanger sequencing (BigDye v3.1, Life Technologies) of the PCR product obtained with the GoTaq[®] G2 Hot Start Master Mix (*Promega*) and purified with ExoSAP (UBS). Sequences were analyzed on a 3730XL sequencer and with the SeqScape software (Life Technologies). Purified PCR products were sequenced using forward and reverse amplification primers (primer sequences are available upon request).

Table 2. Genetic findings

Patient ID	Consang.	Gene	Pathogenic mutation	Variation type	Zygoty ^a	ACMG class ^b	gnomAD (allele count/allele number)	CADD PHRED score ^c	Reference	Approach
P12	NA	RSPH9	NM_152732.5:c.804_806del, p.(Lys268del)	Single amino-acid deletion	Homozygous	5	14/282 846	21.6	[41]	RFLP
P17	Yes	RSPH9	NM_152732.5:c.804_806del, p.(Lys268del)	Single amino-acid deletion	Homozygous	5	14/282 846	21.6	[41]	RFLP
P20	Yes	RSPH9	NM_152732.5:c.804_806del, p.(Lys268del)	Single amino-acid deletion	Homozygous	5	14/282 846	21.6	[41]	RFLP
P1	Yes	CCDC40	NM_017950.2:c.1315C>T, p.(Gln439*)	Nonsense	Homozygous	5	ND	55	[42]	WES
P13	Yes	CCDC40	NM_017950.4:c.2788C>T, p.(Gln930*)	Nonsense	Homozygous	5	ND	41	Novel	WES
P2	Yes	RSPH4A	NM_001010892.2:c.430C>T, p.(Gln144*)	Nonsense	Homozygous	5	3/251 350	33	[20]	WES
P3	No	DNAAF2	NM_018139.3:c.1054_1087del, p.(Ala352Profs*78)	Frameshift	Homozygous	5	ND	25.7	Novel	WES
P6	Yes	ODAD2	NM_018076.5:c.2611-1G>A, p.?	Splice	Homozygous	5	ND	36	Novel	WES
P11	Yes	DNAH5	NM_001369.3:c.958_959dup, p.(Lys322Arfs*4)	Frameshift	Homozygous	5	2/251 054	32	Novel	WES
P16	NA	DRC1	NM_145038.2:c.797_801dup, p.(Asp268*)	Frameshift	Homozygous	5	ND	22.9	Novel	NGS panel
P19	Yes	CCDC39	NM_181426.1:c.2497_2498del, p.(Gln833Valfs*6)	Frameshift	Homozygous	5	4/260 852	26.9	[43]	NGS panel
P14	NA	CFAP300	NM_032930.2: c.(?-33)_(-268 +1_269-1)del, p.?	Deletion	Homozygous	5	ND	NA	Novel	NGS panel
P4	No		No obvious causal variation							WES
P7	Yes		No obvious causal variation							WES
P15	NA		No obvious causal variation							NGS panel
P18	NA		No obvious causal variation							NGS panel

Consang consanguinity, RFLP restriction-fragment length polymorphism, WES whole-exome sequencing, NA not available, ND not described

^aCADD PHRED scores over 20 indicate the 1% most deleterious variations [44]

^bAccording to the American College of Medical Genetics classification (5; pathogenic)

^cHomozygosity was confirmed by sequencing depth ratio analysis that showed two copies of the exon

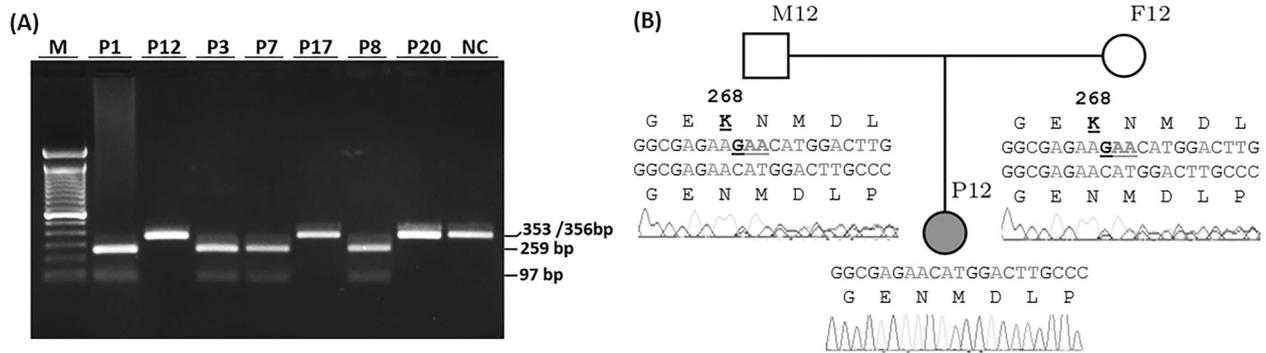


Fig. 1 Screening of the c.804_806del p.(Lys268del) *RSPH9* founder mutation. **A** Electrophoresis of PCR-*MbolI* digestion of exon 5 in probands P1, P12, P3, P7, P17, P14, and P20 (1.5% agarose gel). The founder mutation abolishes the *MbolI* restriction site. P12, P17, and P20 are homozygotes for c.804_806del (non-digested 353 bp band similar to that in the negative control (NC)). Probands P1, P3, P7, and P14 do not carry the mutation. M: 100 bp DNA ladder. NC: PCR amplicon without enzyme. **B** Confirmation of the homozygous genotype in proband P12 and heterozygosity in her parents by Sanger sequencing

RESULTS

Clinical characteristics

Sixteen unrelated patients with clinical suspicion of PCD have been selected in four Pediatrics departments from Saudi hospitals (Jeddah, Makkah, Taif, and Hail). Their median age at diagnosis was one month. Ten of them had a PICADAR score greater than or equal to 5 [range: 3–12]. Five patients had a clinical presentation compatible with Kartagener syndrome. Six patients had NRD without LD (Table 1). Consanguinity was reported in 56% (9/16) of the families (Table 2).

Targeted mutation screening

Two pathogenic founder mutations, respectively in *RSPH9* and *CCDC39*, were screened by PCR-*MbolI* digestion. In 3 out of 11 probands without LD, we identified the pathogenic *RSPH9* founder mutation c.804_806del p.(Lys268del), which confirmed in them the clinical diagnosis of PCD. The three probands were homozygous and their parents were heterozygous (Fig. 1). In contrast, none of the explored probands carried the *CCDC39* c.2190del p.(Glu731Asnfs*31) mutation.

Targeted panel and whole-exome sequencing

By targeted capture panel (encompassing the 48 genes implicated in PCD so far and about 250 candidate genes) and exome sequencing, we have identified biallelic pathogenic mutations in nine additional probands, all of whom were homozygous for distinct mutations. In total, we thus confirmed PCD diagnosis in 75% (12/16) of the probands and characterized six new molecular defects. The ten pathogenic alleles span nine PCD genes (*CCDC39*, *CCDC40*, *CFAP300*, *DNAF2*, *DNAH5*, *DRC1*, *ODAD2*, *RSPH4A*, and *RSPH9*) (Table 2). All mutations were predicted to lead to a premature stop codon, except for the *RSPH9* founder mutation, which leads to a single amino-acid deletion, and the 3-exon deletion in *CFAP300*.

DISCUSSION

Because of its high throughput, NGS has become a precious tool to establish a genetic diagnosis in many hereditary diseases, especially when genetically heterogeneous like PCD. The diagnostic yield of panel-based testing in PCD has been assessed in several studies. In Europe and the United States, sequencing of 18–44 known PCD genes led to diagnostic confirmation in 43–67% of independent cases [19, 35–37]. The sequencing of a panel including 37 PCD-related genes confirmed the diagnosis in 70% of 33 Egyptian independent patients [21]. Among those 33 probands, 28 had LD and/or NRD; 82% of cases in this subgroup (23/28) carried biallelic mutations in a PCD gene. In 34

independent Tunisian probands selected according to more stringent criteria (PICADAR score above 5 and/or ultrastructure defects in TEM and/or low nNO), the diagnostic yield reached 82% with a 41 PCD gene panel [29]. Furthermore, the diagnostic yield of WES-based testing of PCD was evaluated in a number of cohorts. The study of a Turkish cohort of PCD patients through exome sequencing has revealed mutations in 75.4% of cases [38]. In a first study targeting Saudi patients based on clinical suspicion of PCD, the yield of WES was 68% [28]. In this study, we have reached a slightly higher elucidation rate by combining a first-line cost-effective screening of the *RSPH9* ancestral mutation followed by 48-gene panel or exome sequencing (75%). In the end, a molecular cause has been identified in 10 out of the 11 patients with LD and/or NRD.

In concordance with the high proportion of probands born to a consanguineous union and in whom biallelic mutations were identified (75% in solved cases), all of them were homozygous for a pathogenic mutation. This study highlights the genetic heterogeneity of PCD in Saudi patients, as variations have been detected in nine different genes and, except for the founder *RSPH9* mutation found in three unrelated patients, each mutation has been identified in a single family.

The census of all Saudi unrelated patients genetically diagnosed for PCD in previous studies (52 patients) together with the 12 patients in this study shows that 17 out of 64 (3/12 and 14/52) probands (27%) carried the *RSPH9* founder mutation [27, 28, 39–42]. Notably, the high proportion of this mutation among Saudi PCD patients has led to a ratio of Kartagener syndrome much lower than the classically reported 50%, as *RSPH9* encodes a protein belonging to the central complex that is absent from nodal cilia; as a consequence, patients with mutations in *RSPH9* have no LD. In fact, in this study, only 5 among the 12 diagnosed patients (42%) had *situs inversus*. Likewise, LD were reported in 21 of 81 patients (26%) in another Saudi patient group [28]. Of note, the *RSPH9* founder mutation explained 3 out of 11 independent cases without LD in our study, and 41% of independent patients without LD in a large Saudi cohort analyzed by WES [28]. Given that the *RSPH9* allele identified in Saudi families descended from the common ancestor [43], its identification in one Egyptian [21] and one Tunisian PCD patient [29] could be historically explained by the Muslim expansion in North Africa in the seventh century (642–709 AD). Screening first for the c.804_806del p.(Lys268del) founder mutation with the easy-to-access PCR-RFLP technique or Sanger sequencing may thus be helpful in patients of Arabic ancestry who are suspected of PCD and do not show LD.

The *RSPH4A* c.430C>T p.(Gln144*) mutation identified in patient P2 is worth noting. This allele had previously been identified in one Saudi [28], one Turkish [38], and two Egyptian patients [21]. It

has not been reported in other populations. This suggests a founder effect for this variant in the Arabian Peninsula region. On the other hand, the partially shared Arabic ethnic background between North African and Saudi populations prompted us to conduct here targeted testing of the *CCDC39* c.2190del founder mutation frequent in patients originating from Tunisia and identified in other North African countries [29]. Nevertheless, none of the tested Saudi patients carried this variant. Supported by its absence in another study carried out in the same population [28], this result strongly argues that the ancient origin of the *CCDC39* c.2190del allele would be related to Berber populations, which have inhabited North Africa for 3000 years.

The novel *ODAD2* splice mutation (c.2611-1G>A p.?) identified in patient P6 disrupts the acceptor splice site of intron 17 (100% probability for site loss according to SpliceAI prediction) and creates a cryptic site one nucleotide downstream in exon 18 (87% probability predicted by SpliceAI) [33]. The use of the cryptic site would introduce a premature stop codon probably leading to an absence of protein production (through nonsense-mediated mRNA decay) or to a truncated protein p.(Asp871Metfs*20). In addition, we have identified in patient P16 a novel nonsense mutation in *DRC1* (c.797_801dup). Although this gene, encoding a nexin-dynein regulatory complex protein, has been reported to be expressed in mouse nodal cilia during embryogenesis (at 7.5 days of embryonic age) [44], none of the previously reported patients with mutations in this gene, as well as patient P16, had LD.

The four unsolved cases in this study may carry mutations in non-coding regions and/or in a new gene (although WES was performed in two of them). However, some of those cases could have another condition than PCD as 3 of them had a PICADAR score under 5, whereas only 2 of the 12 patients with biallelic mutations had a PICADAR score under 5. Clinical follow-up and further investigations in these negative cases would be instrumental to confirm PCD.

In summary, we show that a sequential approach combining the screening of the founder *RSPH9* mutation followed by NGS analysis targeting the 51 genes implicated so far in PCD (WES or targeted panel) is efficient and cost-effective in patients originating from Saudi Arabia. This work highlights the genetic heterogeneity of PCD in this population, with ten distinct pathogenic mutations—six of which were novel—in 12 families. The availability of other tests (e.g., nasal NO measurement, TEM, or immunofluorescence analysis assessing structural defects in airway cilia) would be helpful to confirm PCD in patients with no LD and no identified mutation. Analysis of other cohorts is valuable to explore further the mutation spectrum of PCD in populations from the Arabic peninsula and North Africa, as well as other populations currently underrepresented in published data.

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COMPETING INTERESTS

The authors declare no competing interests.

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