Biallelic variants in the ciliary gene TMEM67 cause RHYNS syndrome

Biallelic mutations in the ciliary gene *TMEM67* cause RHYNS syndrome

Running Title: Recessive *TMEM67* mutations cause RHYNS syndrome

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Conflict of interests

The authors declare that they have no conflict of interests

ABSTRACT

A rare syndrome was first described in 1997 in a 17-year-old male patient presenting with *R*etinitis pigmentosa, *HY*popituitarism, *N*ephronophthisis and *S*keletal dysplasia (RHYNS). In the single reported familial case, two brothers were affected, arguing for X-linked or recessive mode of inheritance. Up to now, the underlying genetic basis of RHYNS syndrome still remains unknown. Here we applied whole-exome sequencing in the originally described family with RHYNS to identify compound heterozygous mutations in the ciliary gene *TMEM67*. Sanger sequencing confirmed a paternally inherited nonsense (p.Arg208*) and a maternally inherited missense mutation (p.Asp430Gly). Overall, *TMEM67* showed one of the widest clinical continuums observed in ciliopathies ranging from early lethality to adults with liver fibrosis. Our findings extended the spectrum of phenotypes/syndromes resulting from biallelic *TMEM67* mutations to now eight distinguishable clinical conditions including RHYNS syndrome.

Key words: ciliopathy; nephronophthisis; retinitis pigmentosa; *MKS3*; *TMEM67*

INTRODUCTION

RHYNS syndrome (OMIM 602152) was defined in 1997 by the acronym of Retinitis pigmentosa, HYpopituitarism, Nephronophthisis and mild Skeletal dysplasia in a 17-year-old man[1]. A closely resembling phenotype with liver fibrosis has been previously described in a boy[2], while Hedera and collaborators reported a similar condition in a family with two affected brothers[3]. The observation of affected males only and recurrence in a sib suggested either an X-linked or autosomal recessive inheritance pattern. Yet, the underlying genetic basis has remained unexplained, although the pattern of associated clinical features was compatible with a hereditary ciliary disorder. In this study, we reported the 20-year follow-up of the original patient with RHYNS syndrome. Whole-exome sequencing led to the identification of a compound heterozygous mutation in the gene *TMEM67*. The assignment of RYHNS syndrome as part of the wide spectrum of *TMEM67*-related ciliopathies is discussed.

SUBJECTS AND METHODS

Patient

The index male patient was re-evaluated at the age of 38 years, still in search of a molecular diagnosis after receiving a clinical diagnosis of RHYNS syndrome at young age[1]. In brief, he was born with congenital palsy of the III and IV cranial nerve on the right resulting in complete ophthalmoplegia and upper eyelid ptosis and congenital palsy of the VI cranial nerve on the left with exotropia. At the age of 4 years, he measured 90 cm (-4,9SD) and showed delayed bone age. Growth hormone (GH) and thyreotropin releasing hormone (TSH) deficiency were diagnosed and treated with replacement therapy until young adulthood. At this age, radiological examination

showed mild signs of skeletal dysplasia consisting of osteopenia, thin tubular bones, epiphyseal hypoplasia and hypoplastic iliac bones with irregular acetabular margins. At age 11, retinitis pigmentosa and left sensorineural hearing loss were first diagnosed. Abnormal renal function was evident by age 12 years, when a renal biopsy demonstrated a histological pattern consistent with nephronophthisis. Due to the worsening of renal function he underwent a first kidney transplantation from a deceased donor at age 29; but it was rejected. Subsequently, he was started on hemodialysis and, at age 34, a second renal transplantation from deceased donor was performed. Since then, his clinical condition remained stable.

We have evaluated the patient at age 38 years, when he measured 152 cm of height with weight 63,5 kg. Generalized and severe osteoporosis was diagnosed by DEXA examination (femoral head: T-Score -3.5, Z-Score -3.0, BMD 0,458 g/cm^2; lumbar region: T-Score -3.1, Z-Score -3.0, BMD 0,752 g/cm^2). A novel skeletal survey detected moderately shortened long bones, bowed radii, short femoral neck, brachydactyly at hands and feet with more severe involvement of middle phalanges, distal phalanx of the thumbs and metacarpals, moderately thickened calvarium, rotoscoliosis of mild degree, and a posterior arch defect of the sacrum. Diffuse reduction of the bone density with thinning of the diaphyseal cortex, was evident, particularly on hands (Figure 1).

Hormonal dosage showed increased parathyroid hormone (PTH) levels in the blood (164 pg/ml, reference range 14-72 pg/ml), despite having received kidney transplantation. Beside this, all other hormonal levels (including thyroid, pituitary and steroid hormones) were repeatedly checked to be within the normal range. Of note, liver enzymes were repeatedly tested normal as was dedicated liver ultrasound.

Audiometry showed pantonal left-sided moderate-to-severe sensorineural hearing loss. Ophthalmologic examination confirmed no residual visual acuity and complete extinguishment of the electroretinogram in both eyes.
Neuropsychological evaluation excluded functional deficits while brain imaging was normal. He obtained the chartered accountant qualification and he was completely self-sufficient in all daily life activities, although he did not have an employment.

Whole-Exome Sequencing

In order to determine the genetic etiology of the RHYNS syndrome, WES was performed hypothesizing an-underlying autosomal recessive inheritance pattern. Exome capture and next-generation sequencing was carried out at Otogenetics Ltd. (http://www.otogenetics.com) on an Illumina HiSeq2000 (Illumina, San Diego, CA) platform and indexed libraries were subjected to paired-end (2×100 bp read length) sequencing-by-synthesis using fluorescent reversible terminators. Exome enrichment was conducted following the protocol for the SeqCap EZ Human Exome beads (Roche NimbleGen, Inc., Madison, WI, USA). Three µg DNAs, isolated from peripheral blood of the affected patient, his parents and two unaffected brothers were submitted for WES. Sequence reads were mapped to the human reference genome assembly (GRCh37/hg19) using CLC Genomics Workbench™ software (CLC bio, Aarhus, Denmark). Variants were called, filtered, and prioritized according to their pathogenicity scores obtained from the MutationTaster, CADD, and Polyphen-2 web interfaces. Furthermore, variants were cross-referenced with the Human Gene Mutation Database (HGMD, http://data.mch.mcgill.ca/phexdb), and genes known to be implicated in ciliopathy-related disorders were prioritized.

RESULTS

Two mutations in *TMEM67* (NM_153704.5; MIM*609884) were identified in the proband, each inherited from a heterozygous parent, consistent with compound heterozygosity and autosomal recessive inheritance. The two identified mutations were confirmed by Sanger sequencing, namely, a nonsense mutation (c.622A>T, p.Arg208*) in exon 6 (paternal allele), and a missense mutation (c.1289A>G, p.Asp430Gly) near the splice acceptor site of exon 13 (maternal allele). The two healthy brothers were carriers of the nonsense mutation only (Figure 2). The missense variant was absent from the 1000 Genomes Project, the Exome Aggregation Consortium (ExAC, http://exac.broadinstitute.org) and the Genome Aggregation Database (gnomAD, http://gnomad.broadinstitute.org); conversely, the nonsense mutation was present at extremely low frequency in population databases (17 and 49 heterozygous individuals in ExAc and gnomAD, respectively). The nucleotide and deduced protein change were predicted as "disease causing" by the *in silico* pathogenicity prediction program MutationTaster (http://mutationtaster.org) which also predicted for the c.1289A>G a potential alteration of the acceptor splice site. In particular, using the Human Splicing Finder software (http://www.umd.be/HSF3/index.html) this mutation was predicted to abolish the canonical acceptor site with formation of a novel site leading to a shorter (-4 bases) exon 13. The software also predicted the formation of an exonic splicing silencer and the alteration of exonic splicing enhancer.

DISCUSSION

In this study, we report the identification of biallelic mutations in the *TMEM67* gene as

the underlying genetic defect causative of RHYNS syndrome. These findings extend the spectrum of phenotypes resulting from *TMEM67* mutations to now eight distinguishable ciliopathies (Table 1). Their clinical manifestations display a wide range of presentations ranging from lethal phenotypes to patients with organ-specific involvement. Mutations in this gene were initially identified in Meckel syndrome, a lethal disorder displaying central nervous system (CNS) malformations, typically occipital encephalocele, multicystic kidneys, ductal plate dysplasia with congenital hepatic fibrosis (CHF) and postaxial polydactyly[4]. Subsequently, Baala and collaborators identified *TMEM67* mutations in three patients with *pure* (isolated) Joubert syndrome (JS), thus defining the sixth JS locus (JBTS6)[5]. Indeed, different subtypes of JS were associated to *TMEM67* mutations with distinct genotype-phenotype correlations within the spectrum of JS-related disorders (JSRDs), a group of pleiotropic ciliopathies which share in common the Molar Tooth Sign (MTS) at brain imaging[6]. In particular, the strongest correlation was defined with JS and CHF, since around 70% patients affected by so-called COACH syndrome (Cerebellar vermis hypo/aplasia, Oligophrenia, congenital Ataxia, ocular Coloboma, and Hepatic fibrosis) carried biallelic *TMEM67* mutations[7].

In addition to Meckel syndrome and JSRDs, about 10% of patients affected by nephronophthisis (NPHP) and CHF without neurological involvement and normal brain imaging (NPHP11; MIM #613550) had *TMEM67* mutations[8]. Interestingly, the same gene was also mutated in three children with a unique association of polycystic kidney (mimicking autosomal recessive polycystic kidney disease - ARPKD), NPHP, CHF and midbrain-hindbrain abnormalities within the MTS spectrum[9]. More recently, Tarailo-Graovac et al.[10] described a young adult patient with two mutations in *TMEM67*, who

displayed, in addition to the MTS and cerebellar atrophy at brain imaging, mild intellectual disability, adolescent-onset dementia, vertical gaze palsy, ataxia, and progressive hepatic fibrosis, overlapping Niemann-Pick type C manifestations. Lastly, *TMEM67* was mutated in an otherwise healthy adult patient affected by isolated congenital hepatic fibrosis, which represented so far, the mildest end of the *TMEM67*-related spectrum[11].

This intriguing clinical heterogeneity associated with mutations in one and the same gene calls for the delineation of specific genotype-phenotype correlations. The allelic spectrum of *TMEM67* includes missense, truncating and splice site mutations, as well as rare multiexon deletions. Two truncating mutations (either frameshift, nonsense or splice site mutations) occur with high frequency in Meckel syndrome and are not reported in non-lethal phenotypes[12]. Conversely, two missense mutations or a combination of truncating /splicing and missense mutations are prevalent in less severe phenotypes within the JSRD spectrum, i.e. JS and COACH[7]. Hypomorphic mutations in *TMEM67* are associated with NPHP and liver fibrosis (NPHP11), while more than half of these patients display ocular involvement[8]. Interestingly, our patient carried one truncating and one splicing mutation and his phenotype was mainly characterized by retinitis pigmentosa, NPH without any neurologic involvement or liver fibrosis. The absence of either neurologic or hepatic involvement is surprising since these are major manifestations of *TMEM67* mutations (Table 1). Altogether these observations emphasize the role of, yet unidentified, modifier factors in other genes modulating the penetrance of clinical manifestations. Of note, mutations at different loci interacting epistatically under a “multiallelic” inheritance has been proposed as a model for disease penetrance in ciliopathies such as Bardet-Biedl syndrome and nephronophthisis[13,14].

In addition, our patient had hypopituitarism without structural abnormalities of the pituitary gland on brain MRI. Interestingly, growth hormone deficiency was not considered a major feature of JS, but recently two distinct genes (*KIAA0753* and *CELSR2*) were associated with such endocrine anomalies in ciliopathies, strengthening the importance of ciliary function also in the development of the pituitary gland[15,16].

In conclusion, our data place RHYNS syndrome within the spectrum of *TMEM67*-related ciliopathies. This is one of the widest clinical continuums resulting from recessive mutations in a single gene, ranging from early lethality to adults with liver fibrosis. More studies are encouraged to decipher modifier factors influencing the penetrance of clinical manifestations in ciliopathies.

List of abbreviations:

ARPDK: Autosomal Recessive Polycystic Kidney Disease

CHF: congenital hepatic fibrosis

CNS: central nervous system

COACH: Cerebellar vermis hypo/aplasia, Oligophrenia, congenital Ataxia, ocular Coloboma, and Hepatic fibrosis

EDTA: ethylenediaminetetraacetic acid

ExAC: Exome Aggregation Consortium

GH: growth hormone

gnomAD: Genome Aggregation Database

HGMD: Human Gene Mutation Database

JS: Joubert syndrome

JSRDs: Joubert syndrome related disorders

MTS: molar tooth sign

NPHP: nephronophthisis

PTH: parathyroid hormone

RHYNS: Retinitis pigmentosa, HYpopituitarism, Nephronophthisis and Skeletal dysplasia.

TSH: thyreotropin releasing hormone

WES: whole exome sequencing

APPENDIX:

List of the members of the "Undiagnosed Disease Network Italy": Domenica Taruscio (Rome, Italy); Marco Salvatore (Rome, Italy); Maria Chiara De Stefano (Rome, Italy); Federica Censi (Rome, Italy); Giovanna Floridia (Rome, Italy); Francesco Brancati (L'Aquila, Italy); Giuseppe Novelli (Rome, Italy); Erica Daina (Ranica, Italy); Paraskevas Iatropoulos (Ranica, Italy); Alessandra Ferlini (Ferrara, Italy); Marcella Neri (Ferrara, Italy); Dario Roccatello (Turin, Italy); Simone Baldovino (Turin, Italy); Elisa Menegatti (Turin, Italy); Bruno Bembi (Udine, Italy)

Declarations:

Availability of data and materials

All data generated or analyzed during this study are included in this published article.

Authors' contributions

FB and EO had full access to all of the data in the study and take responsibility for the integrity of the data and the accuracy of the data analysis. FB, EO and NP were responsible for the study supervision. LC drafted the manuscript and interpreted the data. EC collected clinical data. VVW performed sequencing analysis. LC, EC and VVW equally contributed to the manuscript. AC is in charge of the patient and contributed relevant clinical data for phenotypic delineation. MC reviewed the skeletal X-Ray images and contributed relevant clinical data for phenotypic delineation. A critical revision of the manuscript for important intellectual content was carried out by FS, GN and NP. UDNI contributed to the administrative, technical and material support. All authors contributed to the study concept and design. All authors were responsible for drafting of the manuscript, contributed to the acquisition, analysis and interpretation

of data, read and approved the final manuscript.

Consent for publication

The participants included in the study signed a written informed consent to publish their data.

Ethics approval and consent to participate

The research protocol, in accordance with the tenets of the Declaration of Helsinki and their reviews, was approved by the Ethics Committee. The patients included in the study signed a written informed consent to participate in the study.

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Titles and legends to figures

Figure 1: Radiological skeletal survey of the proband at age 38 years.

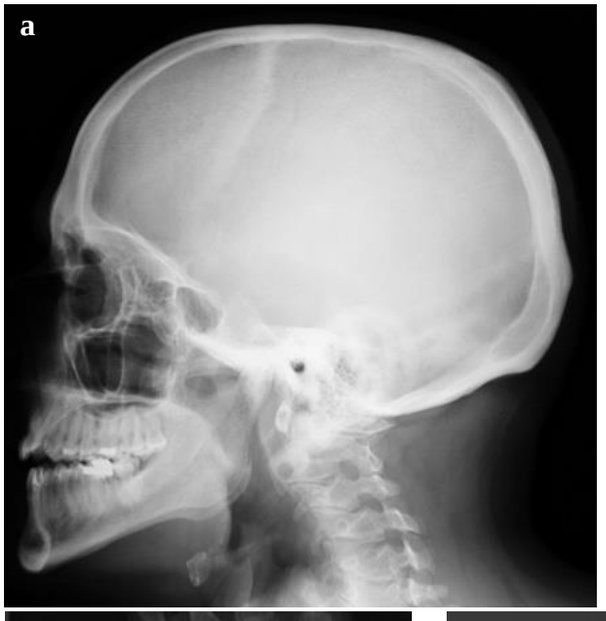
(**a**) Thickened calvarium. (**b**) Short and bowed radius. (**c**) Brachydactyly at hands with more severe involvement of middle phalanges, distal phalanx of the thumbs and metacarpals. Generalized reduction of bone density with thinning of the diaphyseal cortex is observed. (**d)** Rotoscoliosis. (**e**) Posterior arch defect of the sacrum and short femoral necks.

Figure 2: Pedigree of the RHYNS family and *TMEM67* electropherograms.

(**a**) Family tree showing the proband (filled square symbol) and two healthy sibs. Circles and squares indicate females and males, respectively. (**b**) Genomic sequence electropherograms demonstrate a nonsense mutation (c.622A>T, p.Arg208*) in the father (I-1) and all 3 sons (II-1, II-2, II-3) and a missense mutation (c.1289A>G, p.Asp430Gly) in the affected son (II-1) and his mother (I-2). Arrows indicate a compound heterozygous mutation in the affected son and heterozygous changes in all other individuals.

Legend to table:

Table 1: *TMEM67*-related phenotypes and distinctive clinical manifestations.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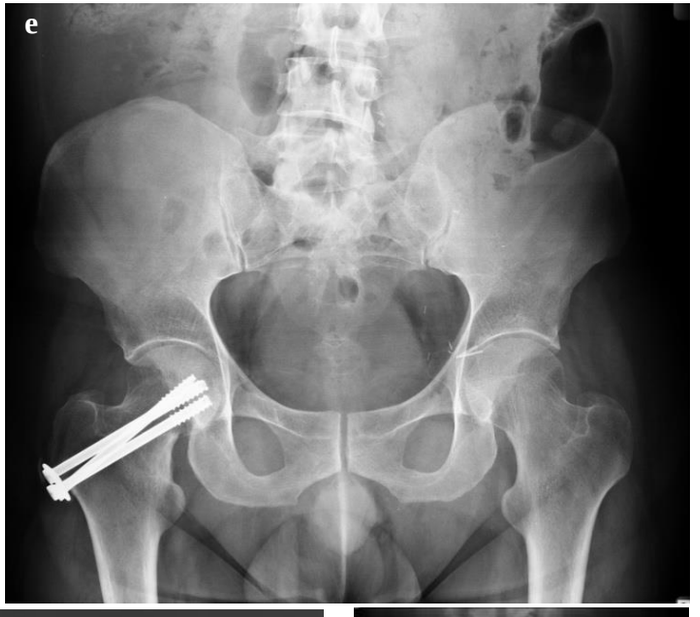
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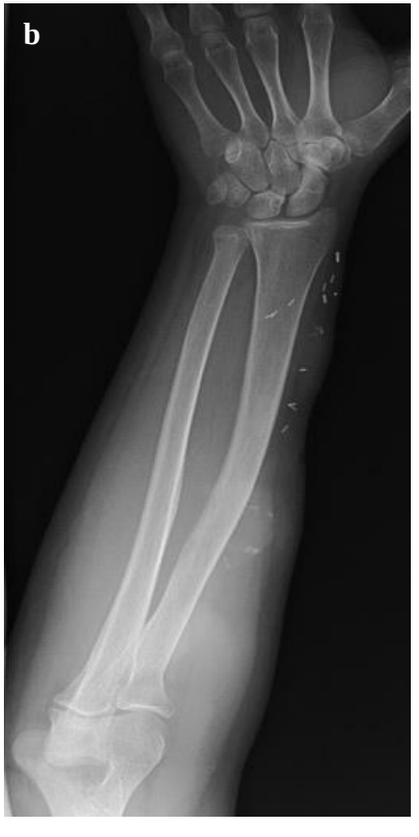
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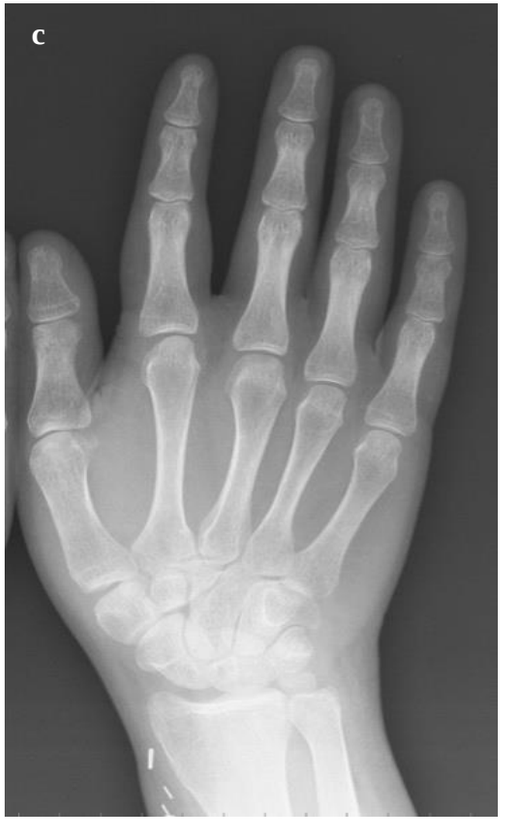
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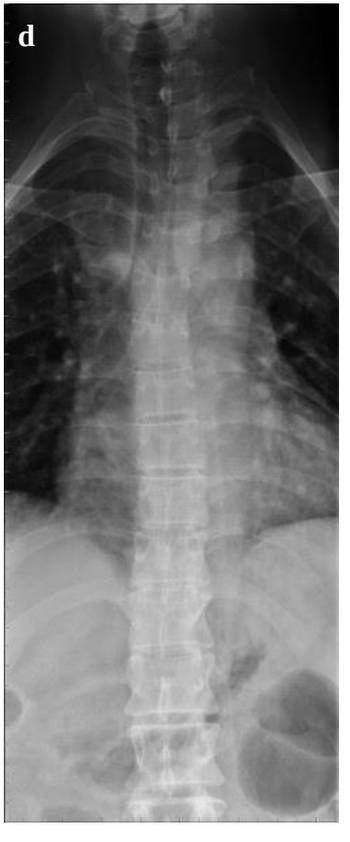
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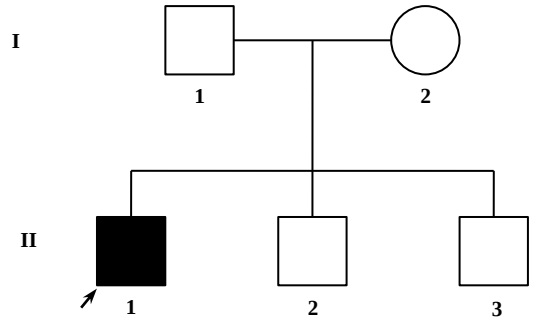

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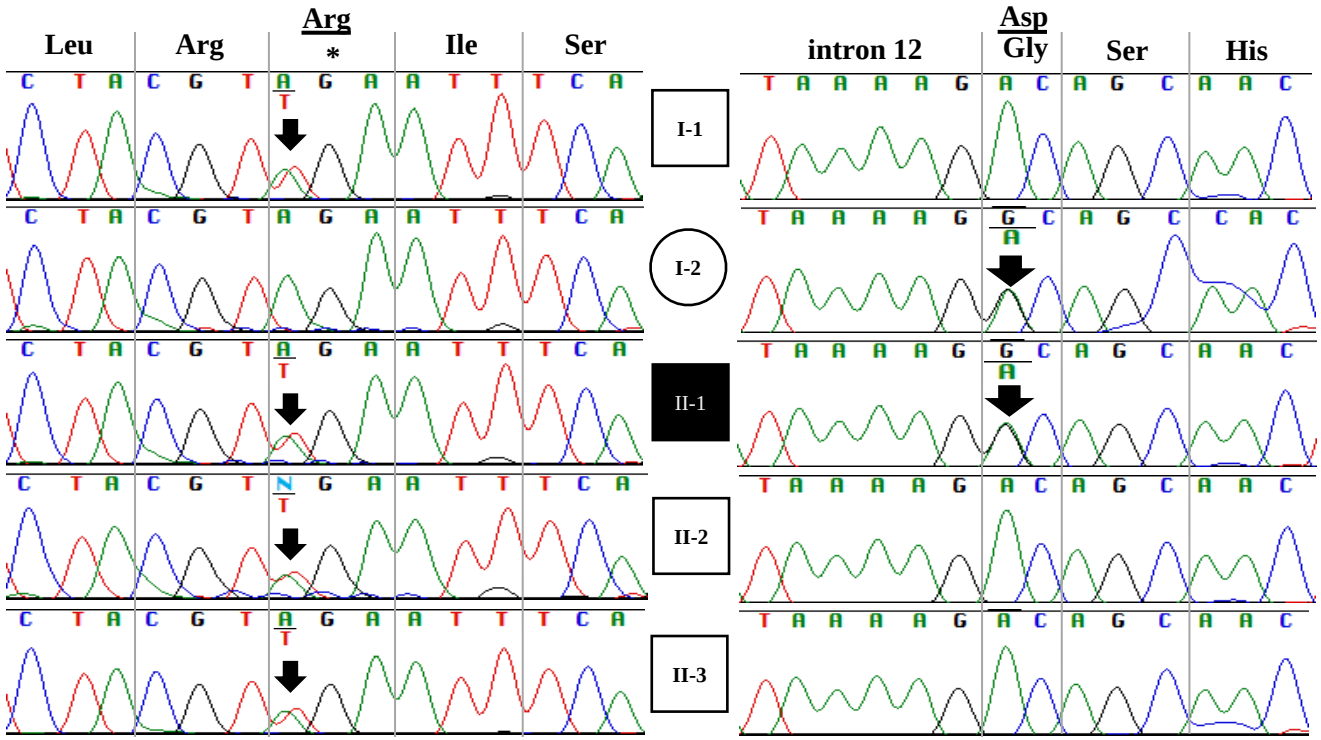

***TMEM67* exon 6**
c.622A>T, p.Arg208*

***TMEM67* exon 13**
c.1289A>G, p.Asp430Gly

Table 1. *TMEM67*-related phenotypes and distinctive clinical manifestations.

Syndrome	Major clinical features	Number of reported patients	Representative references
Meckel syndrome	• Occipital encephalocele • Cystic dysplastic kidneys • Ductal plate malformation • Hepatic fibrosis • Postaxial polydactyly	49	Smith et al.[4] Khaddour et al.[17]
Joubert syndrome	• Molar tooth sign • Intellectual disability (variable) • Hypotonia • Irregular breathing pattern • Eye movement abnormalities	30	Baala et al.[5] Vilboux et al.[6]
COACH	• Molar tooth sign • Intellectual disability (variable) • Ataxia • Ocular coloboma • Hepatic fibrosis • Medullary cystic renal disease • Nephronophthisis	31	Brancati et al.[7] Doherty et al.[18]
NPHP11	• Nephronophthisis • Hepatic fibrosis	8	Otto et al.[8] Srivastavaa et al.[19]
ARPKD-like	• Molar tooth sign-like • Speech apraxia • Polycystic kidneys • Nephronophthisis • Hepatic fibrosis	3	Gunay-Aygun at al.[9]
Niemann-Pick C phenocopy	• Molar tooth sign • Cerebellar atrophy at young age • Intellectual disability • Gaze palsy • Ataxia • Adolescent-onset dementia • Hepatic fibrosis	1	Tarailo-Graovac et al.[10]
Isolated congenital liver fibrosis	• Hepatic fibrosis in an otherwise healthy adult man	1	Vogel et al.[11]
RHYNS	• Retinitis pigmentosa • Gaze palsy • GH- and TSH-deficiency • Nephronophthisis • Skeletal dysplasia • Sensorineural hearing loss	1	This report

Table legend: ARPDK: Autosomal Recessive Polycystic Kidney Disease; COACH: Cerebellar vermis hypo/aplasia, Oligophrenia, congenital Ataxia, ocular Coloboma, and Hepatic fibrosis; GH: growth hormone; NPHP: Nephronophthisis; RHYNS: Retinitis pigmentosa, HYpopituitarism, Nephronophthisis and Skeletal dysplasia; TSH: thyreotropin releasing hormone