BRIEF COMMUNICATION

Clinical variability in *DYNC2H1*-related skeletal ciliopathies includes Ellis-van Creveld syndrome

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Deleterious variants of *DYNC2H1* gene are associated with a wide spectrum of skeletal ciliopathies (SC). We used targeted parallel sequencing to analyze 25 molecularly unsolved families with different SCs. Deleterious *DYNC2H1* variants were found in six sporadic patients and two monozygotic (MZ) twins. Clinical diagnoses included short rib-polydactyly type 3 in two cases, and asphyxiating thoracic dystrophy (ATD) in one case. Remarkably, clinical diagnosis fitted with EvC, mixed ATD/EvC and short rib-polydactyly/EvC phenotypes in three sporadic patients and the MZ twins. EvC/EvC-like features always occurred in compound heterozygotes sharing a previously unreported splice site change (c.6140-5A>G) or compound heterozygotes for two missense variants. These results expand the *DYNC2H1* mutational repertoire and its clinical spectrum, suggesting that EvC may be occasionally caused by *DYNC2H1* variants presumably acting as hypomorphic alleles.

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Ciliopathies are an expanding group of clinically variable and genetically heterogeneous disorders characterized by renal, liver, central nervous system, ocular and skeletal anomalies. Those with predominant skeletal involvement are grouped as skeletal ciliopathies (SCs). SCs comprise Weyers acrofacial dysostosis (WAD, MIM# 193530), Ellis-van Creveld syndrome (EvC, MIM# 225500), cranioectodermal dysplasia (CED, MIM# 218330, or Sensenbrenner syndrome), asphyxiating thoracic dystrophy (ATD, MIM# 208500; or Jeune syndrome), short rib-polydactyly type 1 (SRP1 or Saldino-Noonan type, MIM# 613091), type 2 (SRP2 or Majewski type, MIM# 263520), type 3 (SRP3 or Verma-Naumoff type, MIM# 613091), and type 4 (SRP4 or Beemer-Langer type, MIM# 269860).

To date, at least 30 genes coding for different structural cilia proteins have been implicated in SC [1, 2]. The distinct roles that these proteins have in ciliary function likely underlie some consolidated genotype-phenotype correlations and the different molecular epidemiology among SC. Most EvC cases are due to biallelic variants in *EVC* and *EVC2* [3–5], while a few cases are caused by recessive variants in *WRD35* [6], *DYNC2LI1* [7], *GLI1* [8] or dominant variants in *PRKACA* and *PRKACB* [9]. Among SC-associated genes, *DYNC2H1* is the most commonly involved locus and *DYNC2H1* deleterious variants have been found in a broad spectrum of skeletal ciliopathies ranging from the perinatally lethal SRP types 1, 2, and 3 to non-lethal-ATD cases [1, 2, 10, 11].

We used targeted parallel sequencing to analyze an extended panel of 110 ciliary genes in 26 subjects (7 prenatal and 19 postnatal cases) belonging to 25 families with clinically suspected EvC or another SC. The genes included in the panel are listed in Supplementary Table S1. In all cases, single nucleotide variants in *EVC*, *EVC2*, *WDR35*, *DYNC2LI1*, *GLI1*, *PRKACA* and *PRKACB*, and intragenic copy number variants (CNVs) in *EVC* and *EVC2* had been previously excluded by Sanger sequencing, a restricted multigene panel assessed by parallel sequencing and multiplex ligation-dependent probe amplification analysis. Clinical selection criteria and description of the methods used for the molecular analyses are reported in the Supporting Information.

Sequencing identified putative deleterious variants in *DYNC2H1* (NCBI Reference Sequence: NM_001377.3) in seven index cases, including six sporadic cases and a couple of monozygotic (MZ) twins. In six patients, two variants were identified, while one sporadic case showed a homozygous variant. In patients 1, 3, 6, 7 and 8 parental genotyping confirmed the occurrence of compound heterozygosity. In case 3, the homozygous splice site

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Table 1. Molecular description, population prevalence, bioinformatics predictions, and pathogenicity classification according to the American College of Medical Genetics and Genomics-Association for Molecular Pathology (ACMG-AMP) classification of *DYNC2H1* variants identified in this study.

Patient[a]	Nucleotide substitution[b]	RNA substitution	Protein substitution	Variant type	Protein Domain	GnomAD	CADD score	ΔΔG[c]	ACMG-AMP criteria met	ACMG-AMP classification	Pubmed
1	c.6140-5A>G	r.6139_6140insATAG	p.Val2048ArgfsTer9	Splicing	AAA + 2	Absent	/	NE	PS3_Strong, PM2_Moderate, PM3_Moderate	LP	Not reported
	c.9171_9174delGGAA	NE	p.(Glu3058Ter)	Frameshift	MT-binding stalk	Absent	/	NE	PVS1_VeryStrong, PM2_Moderate. PM3_Moderate	P	Not reported
2	c.12619C>T	NE	p.(Arg4207Ter)	Nonsense	ATP-ase ring (Dynein heavy) C-terminal	0.000004193	56	NE	PVS1_VeryStrong, PM2_Moderate. PM3_Moderate	P	Not reported
	c.6140-5A>G	r.6139_6140insATAG	p.Val2048ArgfsTer9	Splicing	AAA + 2	Absent	/	NE	PS3_Strong, PM2_Moderate, PM3_Moderate	LP	Not reported
3	arr[GRCh37] 11q22.1q22.3 (99715102_103351453) x1	-	-	Whole gene deletion	-	/	/	NE	NA	P	Not reported
	c.6140-5A>G	r.6139_6140insATAG	p.Val2048ArgfsTer9	Splicing	AAA + 2	Absent	/	NE	PS3_Strong, PM2_Moderate, PM3_Moderate	LP	Not reported
4	c.11287G>A	NE	p.(Ala3763Thr)	Missense	AAA + 6	0.00005360	34	−0.808502	PM1_Moderate, PM2_Moderate, PP3_Supporting	VUS	Not reported
	c.3181C>G	NE	p.(Leu1061Val)	Missense	Stem Domain	0.0008700	21.5	0.419578	PM2_Moderate	VUS	Not reported
5	c.11287G>A	NE	p.(Ala3763Thr)	Missense	AAA + 6	0.00005360	34	−0.808502	PM1_Moderate, PM2_Moderate, PP3_Supporting	VUS	Not reported
	c.3181C>G	NE	p.(Leu1061Val)	Missense	Stem Domain	0.0008700	21.5	0.419578	PM2_Moderate	VUS	Not reported
6	c.9171_9174delGGAA	NE	p.(Glu3058Ter)	Frameshift	MT-binding stalk	Absent	/	NE	PVS1_VeryStrong, PM2_Moderate	LP	Not reported
	c.11453C>T	NE	p.(Thr3818Ile)	Missense	AAA + 6	Absent	24.9	1.65291	PM1_Moderate, PM2_Moderate,PM3_Moderate	LP	Not reported
7	c.4699C>G	NE	p.(Leu1567Val)	Missense	Stem Domain	Absent	25.2	1.16155	PM2_Moderate, PM3_Moderate, PP5_Supporting	VUS	1
	c.503-9C>G	r.503_621del	p.(Arg167GlyfsTer4)	Splicing	-	Absent	/	NE	PS3_Strong, PM2_Moderate	LP	Not reported
8	c.1151C>T	NE	p.(Ala384Val)	Missense	Stem Domain	0.0000244	28.3	1.56786	PM2_Moderate, PM3_Moderate PP3_Supporting	VUS	15
	c.6342_6345delTCTT	NE	p.(Phe2114LeufsTer11)	Frameshift	AAA + 2	Absent	/	NE	PVS1_VeryStrong, PM2_Moderate	LP	Not reported

ACMG-AMP American College of Medical Genetics and Genomics-Association for Molecular Pathology, CADD Combined Annotation Dependent Depletion, *GnomAD* Genome Aggregation Database, *LP* likely pathogenetic, *NA* not applicable, *NE* not evaluated, *P* pathogenetic, *VUS* variant of uncertain significance.

[a]*DYNC2H1* mutations have been included in the LOVD database (https://databases.lovd.nl/shared/variants/DYNC2H1/unique); LOVD individual IDs: patient 1 (#0000407149), patient 2 (#0000407151), patient 3 (#0000407152), patient 4 (#0000407195), patient 5 (#0000407195), patient 6 (#0000407196), patient 7 (#0000407197); patient 8 (#0000409854).

[b]NCBI Reference Sequence: NM_001377.3.

[c]ΔΔG (ΔGmt − ΔGwt) for DYNC2H1 mutant protein.

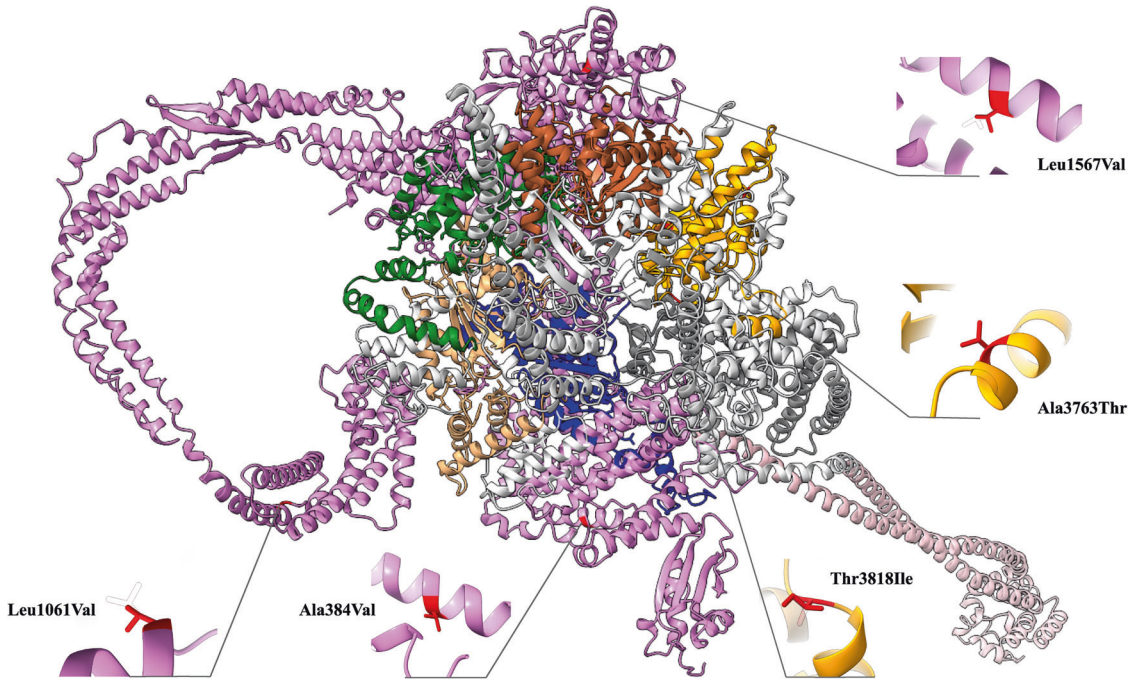

Fig. 1 3D structure of DYNC2H1 obtained through modeling. Functional domains were colored in violet (stem, residues 1-1650), brown (AAA + 1, residues 1651–1875), green (AAA + 2, residues 1938–2161), beige (AAA + 3, residues 2251–2505), blue (AAA + 4, residues 2617–2863), pink (stalk, residues 2881–3169), gray (AAA + 5, residues 3244–3473) and orange (AAA + 6, residues 3690–3905). Variants were mapped on the wild-type structure and highlighted in red.

change, c.6140-5A>G, was absent in the father, whose paternity had been confirmed by DNA fingerprinting test (PowerPlex 16 System, Promega, Madison, WI, USA). In this patient, SNP-array analysis allowed to identify a large deletion encompassing 31 genes, including the entire *DYNC2H1* as well as other 21 OMIM genes (arr[GRCh37] 11q22.1q22.3 (99715102_103351453) × 1) (Supplementary Fig. S1 and Supplementary Table S2).

The mutation spectrum included a total of 11 *DYNC2H1* variants (Table 1). Among the novel variants, two were recurrent (c.6140-5A>G [patients 1, 2, and 3] and c.9171_9174delGGAA [patients 1 and 6]). Although the *DYNC2H1* variants were distributed along the entire length of the gene, missense changes affected two specific regions of the protein, the Stem and AAA + 6 domains (Fig. 1). We explored a possible structural and functional impact of these amino acid alterations in terms of protein stability by measuring their induced thermodynamic change [12]. Based on the difference in free energy value (ΔΔG = ΔGmt − ΔGwt), p.(Ala384Val), p.(Leu1567Val) and p.(Thr3818Ile) variants were classified as destabilizing and p.(Ala3763Thr) change as slightly stabilizing. Finally, a neutral impact was predicted for the p.(Leu1061Val) substitution. The free energy calculations results are summarized in Table 1, while protein stability study methods are described in the Supporting Information.

According to in silico splicing predictions, c.6140-5A>G and c.503-9C>G intronic variants were expected to affect proper transcript processing (Supporting Information). cDNA analysis confirmed that both variants affected splicing. Specifically, c.6140-5A>G creates a new 3′ splice acceptor site leading to an aberrantly processed transcript, which incorporates four bases of intron 38 (r.6139_6140insATAG) (Supplementary Fig. S2). This altered processing results in a frameshift and introduces of a premature termination codon [p.(Val2048ArgfsTer9)], which is predicted to lead to nonsense-mediated mRNA decay (NMD). Similarly, cDNA analysis confirmed that also c.503-9C>G affects splicing by causing the out-of-frame skipping of exon 4 (r.503_621del), the consequent introduction of a premature stop codon, and the translation of a truncated protein presumably undergoing NMD (Supplementary Fig. S3).

Demographic, radiographic and clinical data of the eight patients are shown in Table 2. Selected clinical and radiological features are illustrated in Supplementary Fig. S4. Detailed clinical descriptions of each case are reported in Supporting Information. In summary, clinical features suggested the diagnosis of EvC in two cases (patients 2 and 4/5), SRP3 in two cases (patients 6 and 7), ATD in one case (patient 1) and a mixed EvC/ATD (patient 3) or EvC/SRP3 (patient 8) phenotype in two cases.

Deleterious biallelic variants in *DYNC2H1* gene have been identified in patients with ATD, SRP1, SRP2, SRP3, and very recently in three individuals with EvC showing no cardiac involvement, but multiple frenula and nail hypoplasia [13]. The present results further expand *DYNC2H1*-associated mutational repertoire and widen the clinical spectrum of the deleterious variants of this gene to include also EvC. The identification of potentially disease-causing *DYNC2H1* variants in individuals with a presentation fitting with EvC rather than with ATD and short rib-polydactyly is not surprising within the spectrum of SCs. The current nosology of hereditary bone disorders maintains separate these conditions [14]. Such a distinction is supported by considering the overall severity, postnatal life expectancy and pattern of associated extra-skeletal features. However, the existence of a phenotypic continuum among them dates back to the observation of SRP3 and ATD in the same family [15]. In the present cohort, two index cases had a diagnosis of SRP3 before molecular testing, supporting the presence of a predominant pattern of anomalies associated with *DYNC2H1* deleterious variants at the severe end of the spectrum. In addition, biallelic *DYNC2H1* variants were found in a case of ATD with multiple oral frenula and favorable prognosis (patient 1). A sporadic case was considered affected by a mixed ATD/EvC phenotype featuring short stature of the short limb type, dysplastic nails, tetramelic postaxial polydactyly, and congenital heart and genitourinary anomalies (patient 3). Another sporadic case was clinically framed as a mixed SRP3/EvC phenotype

Table 2. Clinical features in patients with biallelic *DYNC2H1* variants compared with those characterizing Ellis-van Creveld syndrome (EvC), asphyxiating thoracic dystrophy (ATD) and short-rib thoracic dysplasia type 3 (SRP3).

Feature	1	2	3	4*	5*	6	7	8	ATD	EvC	SRP3
Sex	F	M	M	F	F	M	F	M			
Country of origin	Italy	Italy	Italy	Italy	Italy	Albany	Italy	Italy			
Consanguineus parents	-	-	-	-	-	-	-	-			
Age	1 month	NA	4 month	NA	NA	NA	NA	1.9 years			
Oral frenula	+	HLF	-	-	-	-	-	-	-	+	-
Dental anomalies	-	SSCT	-	HD, CT, EH	HD, CT, EH, MO	-	-	-	-	+	-
Dysplastic nails	-	-	STN	+	+	-	-	-	-	+	-
Short stature	+	+	+	+	+	+	+	-	+	+	+
Narrow thorax	+	+	+	+	+	+	+	+	+	+	+
Limb shortening	+	+	+	+	+	+	+	-	+	+	+
Irregular metaphyses	+	-	-	-	-	-	-	-	+	-	+
Vertebral anomalies	-	-	-	-	-	FVB	-	-	-	-	P
Pelvic abnormalities	-	-	-	-	-	SIWSLS	-	+	+	+	+
Hand postaxial polydactyly	B	B	B	B	B	+	+	-	Rare	+	Rare
Feet postaxial polydactyly	-	U	B	U	B	+	+	-	Rare	+	Rare
Hand brachydactyly	-	-	+	-	-	-	-	+	+	-	+
Hand/feet sindactyly	-	-	+	-	-	-	-	+	+	-	+
Cardiac defect	-	-	HLVA	pAVC, PDA	pAVC, PDA	-	-	-	TGA	+	-
Renal anomalies	-	-	HK, MCK	-	-	RD	HK	-	+	-	+
Pulmonary anomalies	PI	-	-	-	-	NA	NA	-	+	-	+
Gastrointestinal anomalies	-	-	-	-	-	IM	-	-	-	-	IM
Other anomalies	-	IH, PolyH	-	-	-	-	-	CF			
Clinical diagnosis	ATD	EvC	EvC/ATD	EvC	EvC	SRPS3	SRPS3	EvC/SRP3			

+, present; -, absent; *, monozygotic twins.

B bilateral, *BH* bilateral hydronephrosis, *CF* club foot, *CT* conical teeth, *EH* enamel hypoplasia, *F* female, *FVB* flattened vertebral bodies, *HD* hypodontia, *HK* hyperechogenic kidneys, *HLF* hypertrophic labiogingival frenulum; *HLVA* hypertrabeculature of left ventricle apex, *IH* inguinal hernia, *IM* intestinal malrotation, *M* male, *MCK* multicistic kidneys, *MO*, malocclusion, *NA* not available; *P* platyspondyly, *pAVC* partial atrioventricular canal, *PDA*, patent ductus arteriosus, *PI* pulmonary insufficiency *PolyH* polyhydramnios, *RD* renal dysplasia, *SIWSLS* small iliac wings with small lower spur, *SSCT*, small, sharp and conical teeth, *STN* short thin nails, *U* unilateral, *TGA* great vessels transposition, *VC* vertebral cleft.

because of a mild bone dysplasia with short ribs associated with partial feet syndactyly. Finally, two MZ twins showed full-blown characteristics of EvC including multiple oral frenula, dysplastic nails, short stature with short limbs, narrow thorax and polydactyly (patients 4 and 5). The present findings support the existence of a much wider phenotypic spectrum for biallelic *DYNC2H1* variants extending to an attenuated skeletal phenotype strongly resembling EvC.

We identified 11 different *DYNC2H1* variants, including two known as pathogenic [1, 16], and nine not previously reported. To our knowledge, this is the first description of compound heterozygosity for a whole gene deletion (WGD) including *DYNC2H1*. Interestingly, the novel c.6140-5A>G splice site change was found in three subjects with variable clinical features fitting with ATD (case 1), EvC (case 2), and both conditions (case 3). In two cases, c.6140-5A>G was combined with a truncating variant [p.(Glu3058Ter), p.(Arg4207Ter)], and in one case with a WGD. We speculate that the phenotypic variability of these patients, ranging from ATD to EvC, is attributable to a variable loss of DYNC2H1 function induced by the null allele, while the specific c.6140-5A>G splice change probably acts as a hypomorphic allele. Accordingly, this intronic variant has never been identified in association with SRP3, which is the most severe *DYNC2H1*-related SC. c.6140-5A>G was found in patients from Southern Italy, probably representing a founder effect in that population. Interestingly, the three *DYNC2H1*-mutated individuals with EvC phenotype recently described in Aubert-Mucca et al. [13] were also compound heterozygous for a mutant null allele and an intronic variant outside the canonical splice sites. The description of further *DYNC2H1*-mutated EvC cases may clarify whether or not there is an association between *DYNC2H1* intronic variants and EvC. The identification of two missense changes [p.(Ala3763Thr) and p.(Leu1061Val)] in MZ twins displaying a typical EvC phenotype may suggest the existence of specific *DYNC2H1* missense variants determining less severe consequences on the protein function and, thus, leading to milder phenotypes. This hypothesis seems to be consistent with protein stability computations, predicting that variants associated with SRP3 destabilize the protein [p.(Ala384-Val), p.(Leu1567Val) and p.(Thr3818Ile)], while those associated with EvC are either neutral [p.(Leu1061Val)] or increase protein stability [p.(Ala3763Thr)].

In conclusion, this study adds nine novel variants to the *DYNC2H1* mutational repertoire and provides evidence that the associated clinical spectrum of pathogenic *DYNC2H1* variants includes EvC and EvC-like phenotypes. This work also highlights the utility to add splicing and CNV analysis in the diagnostic flowchart of SCs in order to improve the clinical effectiveness of the laboratory report.

DATA AVAILABILITY

The datasets generated and/or analyzed during the current study are available from the corresponding author on reasonable request. All variants have been added to the Leiden Open Variation Database (LOVD, https://databases.lovd.nl/shared/variants/DYNC2H1/unique).

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AUTHOR CONTRIBUTIONS

Conceptualization: FP-S, BM, MT, ADL; Formal analysis: MCD, FP, LB, TM; Funding acquisition: FP-S, LM, TM, MT, MC, ADL; Investigation: FP-S, LM, BT, VG, FC, IT, AO, EF, MCD, FP, LB, CM, DC, ADL; Project administration: FP-S, ADL; Resources: FS, DG, GM, CP, FF, NB-P, AN, AP, BM, MCD, BD, VLR-P, MC; Supervision: TM, MC, ADL; Visualization: FP-S, LM, LB, MCD, TM, ADL; Writing – original draft: FP-S, LM; Writing – review & editing: TM, BD, VLR-P, MT, MC, ADL.

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COMPETING INTERESTS

The authors declare no competing interests.

ETHICS APPROVAL

The study was performed in accordance with the principles set out in the 1984 Declaration of Helsinki and subsequent versions and was approved by the local institutional review board (no. 13/CE 2021). All patients signed an informed consent for the scientific use of clinical and genetic data. Written informed consent for publication of images was obtained.

ADDITIONAL INFORMATION

Supplementary information The online version contains supplementary material available at https://doi.org/10.1038/s41431-022-01276-7.

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