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SHORT REPORT

Identification of bi-allelic *LFNG* variants in three patients and further clinical and molecular refinement of spondylocostal dysostosis 3

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Abstract

Spondylocostal dysostosis (SCD), a condition characterized by multiple segmentation defects of the vertebrae and rib malformations, is caused by bi-allelic variants in one of the genes involved in the Notch signaling pathway that tunes the "segmentation clock" of somitogenesis: *DLL3*, *HES7*, *LFNG*, *MESP2*, *RIPPLY2*, and *TBX6*. To date, seven individuals with *LFNG* variants have been reported in the literature. In this study we describe two newborns and one fetus with SCD, who were found by trio-based exome sequencing (trio-ES) to carry homozygous (c.822-5C>T) or compound heterozygous (c.[863dup];[1063G>A]) and (c.[521G>T];[890T>G]) variants in *LFNG*. Notably, the c.822-5C>T change, affecting the polypyrimidine tract of intron 5, is the first non-coding variant reported in *LFNG*. This study further refines the clinical and molecular features of spondylocostal dysostosis 3 and adds to the numerous

investigations supporting the usefulness of trio-ES approach in prenatal and neonatal settings.

KEYWORDS

exome sequencing, *LFNG*, neonatal, notch signaling pathway, prenatal, respiratory distress, splicing, spondylocostal dysostosis

1 | INTRODUCTION

Spondylocostal dysostosis (SCD), a condition characterized by multiple segmentation defects of the vertebrae (SDV) and rib malformations, is caused by bi-allelic variants in one of the genes involved in the Notch signaling pathway, which tunes the "segmentation clock" of somitogenesis: *DLL3*, *HES7*, *LFNG*, *MESP2*, *RIPPLY2*, and *TBX6*.[1] Molecular defects in these genes are found in 20%–25% of SCD individuals, with *DLL3* representing the major causative gene.[2,3] Unfortunately, most of the individuals with a clinical diagnosis of SCD remain without molecular confirmation, suggesting further genetic heterogeneity.

Due to the limited number of reported SCD cases, a precise genotype–phenotype correlation is lacking. In a study of 73 individuals with SDV, the two *LFNG*-mutated individuals presented a more severe phenotype, resulting in angulated vertebral bodies and remarkable shortening of the spine.[2] In the literature, only seven individuals with *LFNG* variants have been documented so far.[2–8] We describe five unreported *LFNG* variants (one homozygous and four compound heterozygous) that were identified in diagnostic settings by trio-based exome sequencing (ES) in two newborns and one fetus with definitive diagnosis of SCD type 3 (SCDO3 [MIM: 609813]).

2 | CLINICAL PHENOTYPES

Genotypic and clinical phenotypic data from individuals with *LFNG* variants were recruited through a collaborative network. All individuals were carefully evaluated by a multidisciplinary team of gynecologists, neonatologists, pediatricians, and clinical geneticists of their respective referral center. Clinical information related to prenatal and postnatal growth parameters, dysmorphology, neurodevelopment, skeletal development, cardiac and respiratory functions, and recurrent infections were collected. Written informed consent for diagnostic genetic testing and publication of the clinical information was obtained from the parents of each research subject according to the Declaration of Helsinki.

The age of individuals included in this study, all females, ranged from 1 month to 5 years (Table 1). One individual (proband 3) was born to first-degree Moroccan consanguineous parents. Skeletal anomalies, including hemivertebrae and rib fusion, were detectable already in the prenatal period (Table 1 and Figure 1A–D). After birth, all individuals were admitted to the neonatal intensive care unit (NICU) because of respiratory distress, which was lethal in one case (proband 1). More clinical details are available in the Supporting Information.

3 | GENETICS FINDINGS

Karyotyping and array-CGH were normal in all probands, whereas trio-ES identified *LFNG* bi-allelic variants in all of them. No other potentially relevant variants were identified, especially in genes related to abnormality of the skeletal system/skeletal dysplasia in the Human Phenotype Ontology (HPO) (HP:0000924; HP:0002652) and PanelApp (Skeletal dysplasia, v3.11).

Compound heterozygous variants were detected in probands 1 and 2: c.[863dup];[1063G>A], p.[(Asp289*)];[(Asp355Asn)] and c.[521G>T];[890T>G], p.[Arg174Leu];[Val297Gly], respectively (Figure 1A, Family 1 and Family 2). According to the ACMG/AMP and ACGS guidelines for the interpretation of sequence variants,[9,10] the c.863dup nonsense variant, inherited from the mother of the proband 1, was classified as pathogenic, whereas the paternally inherited c.1063G>A missense substitution as variant of uncertain significance (VUS). According to the GnomAD v3.1.2 database (https://gnomad.broadinstitute.org/), the first variant is unreported, while the latter is extremely rare also in TOPMed/BRAVO datasets (https://bravo.sph.umich.edu/freeze8/hg38/) and considered damaging by several in silico predictors (Table 1). In the proband 2, the c.521G>T change, inherited from the father, is extremely rare in GnomAD v3.1.2 while the maternally inherited c.890T>G substitution is absent in GnomAD v3.1.2 and TOPMed/BRAVO. Both variants are classified as VUS and considered damaging by several in silico predictors (Table 1).

In the proband 3, ES on chorionic villi identified a homozygous intronic variant located five nucleotides upstream of the splice acceptor site of the *LFNG* exon 6: c.822-5C>T (Figure 1A, Family 3). The variant, which is present in both parents in the heterozygous state (DNA from the healthy sibling was not available for testing), has an extremely low frequency in GnomAD v3.1.2 and TOPMed/BRAVO, where no homozygotes are reported, while it is absent in the "al mena" database (https://clingen.igib.res.in/almena/), the largest genome repository in the Middle Eastern and North African (MENA) region, as well as in the GME (Greater Middle East) Variome Project (http://igm.ucsd.edu/gme/) and our in-house database of ~6000 samples. The c.822-5C>T variant was classified as VUS. This nucleotide change affects the polypyrimidine motif, which is necessary for splicing processes.[11] Computational tools predicted no (or eventually

TABLE 1 List of reported biallelic variants of *LFNG* in individuals with SCD3

				Clinical phenotype		Variant				
Reference (PMID)	Age	Sex	Origin	Skeletal features	Other features	*LFNG* variant*	Zygosity**	GnomAD v3.1.2	Bravo/TopMed	Affected domain
Sparrow et al[4] (PMID: 16385447)	n.a.	M	Lebanon	Severe vertebral segmentation anomalies Nonprogressive scoliosis of cervical and thoracic spine, severe foreshortening of the spine, "pebble beach" sign	Long and slender fingers, camptodactyly	c.564C>A p.(Phe188Leu) (exon 3)	Hom	-	-	Fringe
Lefebvre et al[2] (PMID: 29459493)	n.a.	n.a.	Caucasian	Multiple segmentation defects of the vertebrae Angulated vertebral bodies Severe shortening of the spine		c.583T>C p.(Trp195Arg) (exon 4)	Comp.het	-	-	Fringe
						c.842C>A p.(Thr281Lys) (exon 6)		-	-	Fringe
Lefebvre et al[2] (PMID: 29459493)	n.a.	n.a.	Caucasian	Multiple segmentation defects of the vertebrae Angulated vertebral bodies Severe shortening of the spine		c.44dupG p.(Ala16Argfs*135) (exon 1)	Hom	-	-	Transmembrane helical
Maddirevula et al[5] (PMID: 29620724) Shamseldin et al[6] (PMID: 34645488)	Newborn	F	Arab	Multiple hemivertebrae and butterfly vertebrae at the upper dorsal and lumbar spine (8 pairs of ribs) Reduced number of the vertebral bodies at lumbar and sacral spine Significant shortening of the vertebral column Asymmetry of the thoracic cage and mild enlargement of the iliac wings Widening of the atlantoaxial distance J-shaped sella		c.761C>T p.(Thr254Met) (exon 5)	Hom	-	-	Fringe
Takeda et al[3] (PMID: 30196550)	16 y	M	Japan	Severe scoliosis Multiple vertebral and rib malformations (hemivertebrae, block and butterfly vertebrae, fused and hypoplastic ribs)	Short stature (−2.1 SD)	c.467T>G p.(Leu156Arg) (exon 2)	Comp.het	-	4/250394 (no hom)	Fringe
						c.856C>T p.(Arg286Trp) (exon 6)		1/152190 (no hom)	-	Fringe
Otomo et al[8] (PMID: 30531807)	9 m	M	Japan	Multiple vertebral anomalies (rib fusion, "pebble beach" appearance of the vertebral bodies) Malalignment (kyphosis) and hypoplastic odontoid process	Short stature (−2.5 SD) Inguinal herniation	c.372delG p.(Lys124Asnfs*21) (exon 1)	Comp.het	-	1/248830 (no hom)	Fringe
						c.601G>A p.(Asp201Asn) (exon 4)		-	-	Fringe

TABLE 1 (Continued)

				Clinical phenotype		Variant				
Schuhmann et al[7] (PMID: 33728697)	17 y		n.a.	Severe segmentation defects of vertebrae of thoracic and lumbar spine (hemivertebrae, butterfly vertebrae) Severe kyphoscoliosis with dysplastic spine and rib anomalies (rib fusion, rib aplasia/dysplasia)	Ectopic kidney Uterine dysgenesis Mayer-Rokitansky-Küster-Hauser syndrome Absence epilepsy Inner ear deafness	c.446C>T p.(Thr149Ile) (exon 2)	Hom	-	-	Fringe
Current study (Proband 1)	1 m	F	Algeria	Rib fusion, vertebral schisis, hemivertebrae Kyphoscoliosis Thoracic dysplasia Hypoplastic coccyx	Polyhydramnios Bradycardia Respiratory distress Generalized hypotonia	c.863dupC p.(Asp289Ter) (exon 6) c.1063G>A p.(Asp355Asn) (exon 7)	Comp.het	- 7/279880 (no hom)	- 1/125568 (no hom)	Fringe Fringe
Current study (Proband 2)	3 y	F	Italy	Hemivertebrae, vertebral fusion Reduced number of ribs (9 pairs) "Pebble beach" sign with irregular and slightly rounded vertebral bodies Scoliosis Bilateral knee valgus	Short stature (−2.62 SD) Bradycardia Respiratory distress Gastroesophageal reflux Unilateral hearing loss Joint laxity	c.521G>T p.(Arg174Leu) (exon 3) c.890T>G p.(Val297Gly) (exon 6)	Comp.het	1/152244 (no hom) -	- -	Fringe Fringe
Current study (Proband 3)	5 y	F	Morocco	Spinal dysraphism / severe shortening of the spine Multiple vertebral anomalies (hemivertebrae, butterfly vertebrae), rib fusion Hypoplastic coccyx Short neck Pectus carinatum	Short stature Low weight Respiratory distress Diaphragmatic hernia Long slender fingers	c.822-5C>T (intron 5)	Hom	4/152208 (no hom)	5/125568 (no hom)	

Pathogenicity prediction[****]								
ACMG classification[***]	ClinVar HGMD	DANN	SIFT	PROVEAN	Mutation Taster	CADD score	GERP score	PhyloP100 score
Pathogenic (PS1, PM2, PP3)	Pathogenic (#6999) CM060049	Damaging	Damaging	Damaging	Disease causing	31	4.9290	3.7379
"Tepid" VUS (PM2, PP3)	- CM189327	Damaging	Damaging	Damaging	Damaging	31	5.32	5.7989
"Tepid" VUS (PM2, PP3)	- CM189328	Damaging	Damaging	Damaging	Disease causing	25	4.6999	7.3959
Likely pathogenic (PVS1, PM2)	- CI189329						3.2699	

(Continues)

TABLE 1 (Continued)

Pathogenicity prediction[****]								
"Tepid" VUS (PM2, PP3)	- CM1821694	Damaging	Damaging	Damaging	Disease causing	33	5.3099	7.19
"Tepid" VUS (PM2, PP3)	- CM1812798	Damaging	Damaging	Damaging	Disease causing	25.7	4.8299	7.0209
"Tepid" VUS (PM2, PP3)	- CM1812799	Damaging	Damaging	Damaging	Disease causing	28.7	4.6999	5.5339
Pathogenic (PVS1, PS3, PM2)	Pathogenic (#619140) CD193731						4.19	
Likely pathogenic (PS3, PM2 PM3, PP3)	Pathogenic (#619139) CM193732	Damaging	Damaging	Damaging	Disease causing	29.9	5.32	9.394
"Tepid" VUS (PM2, PP3)	- CM2111403	Damaging	Damaging	Damaging	Disease causing	24.2	4.8299	6.761
Pathogenic (PVS1, PM2)	- -						4.6999	
"Hot" VUS (PM2, PM3, PP3)	- -	Damaging	Damaging	Damaging	Disease causing	26.9	4.46	9.2799
"Tepid" VUS (PM2, PP3)	- -	Damaging	Damaging	Damaging	Disease causing	31	5.1799	9.198
"Tepid" VUS (PM2, PP3)	- -	Damaging	Damaging	Damaging	Disease causing	29.2	4.6999	7.5
"Tepid" VUS (PM2, PP3)	- CS2010966	Tolerated			Disease causing	0.455	6.46	4.46

[a]Transcript reference sequence: NM_001040167.2; Protein: NP_001035257.1.
[b]"Hom" = Homozygous, "Comp.het" = Compound heterozygous.
[c]ACMG classification according to Richards et al[9] and Ellard et al.[10].
[d]CADD: 0–50 (threshold of deleteriousness >20), GERP: −12.3 to 6.17, PhyloP: −20.0 to 10.003.

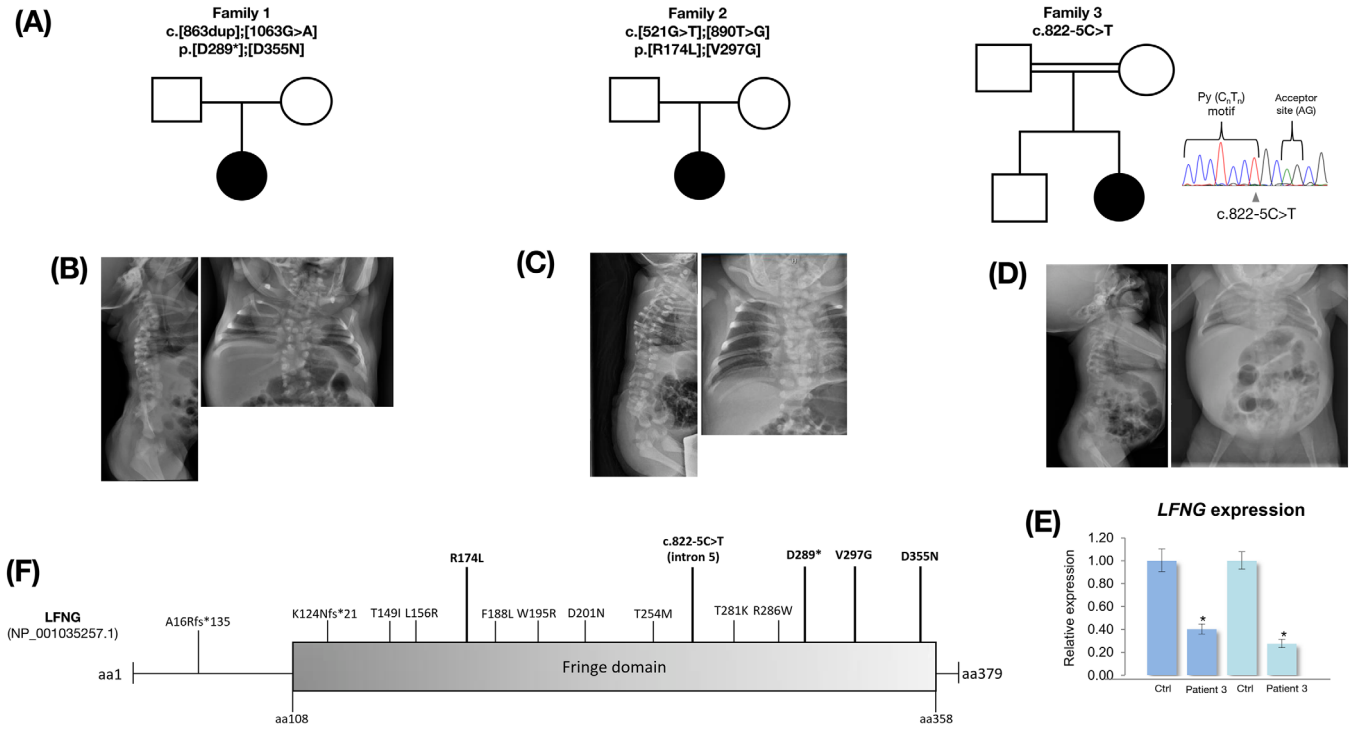

FIGURE 1 (A) Pedigrees and *LFNG* variants identified in families 1, 2 and 3. In proband 3, the variant falls within the polypyrimidine (Py) tract prior to the splice acceptor site (AG) of the *LFNG* exon 6. (B–D) Spinal (left panels) and chest (right panels) X-rays showing diffuse costal and vertebral anomalies in probands 1 (B), 2 (C) and 3 (D). (E) *LFNG* expression in proband 3 was analyzed by RT-qPCR using two primer pairs (represented in light and dark blue) and normalized to *GAPDH*. Data are expressed as mean ± SD from a representative experiment with samples run in triplicate and analyzed using the $2^{-\Delta\Delta Ct}$ method. As control (ctrl) an age- and sex-matched LCL obtained from a healthy individual was used. Comparable results were also obtained by using a commercial cell line (Coriell LCL #GM22647). $^{*}p < .05$ (Student's *t*-test). (F) Schematic representation of the beta-1,3-*N*-acetylglucosaminyltransferase lunatic fringe protein encoded by *LFNG* and distribution of published variants in individuals with SCDO3 [Colour figure can be viewed at wileyonlinelibrary.com]

slight) effects on the splicing, as experimentally confirmed by RT-PCR and sequencing of the *LFNG* cDNA obtained from the patient's lymphoblastoid cell line (LCL) by using primer pairs spanning exons 3–8 (Figure S1). On the other hand, SFmap (https://sfmap.technion.ac.il/) predicted changes of binding sites in the mutant sequence, particularly the formation of two binding sites for the RNA-binding protein Celf1 (CUG-BP, Elav-like family member 1; Figure S2). Under the hypothesis that the predicted novel binding sites for Celf1 could perturb the expression of *LFNG*, a quantitative PCR was performed on the LCL' cDNA, revealing a significantly reduced expression of *LFNG* (Figure 1E).

All variants have been submitted to LOVD v.3.0 (Leiden Open Variation Database; https://databases.lovd.nl/shared/genes/LFNG) with the following accession numbers (DB-ID): LFNG_000025 (c.521G>T); LFNG_000026 (c.822-5C>T); LFNG_000027 (c.863dup); LFNG_000028 (c.890T>G); LFNG_000029 (c. 1063G>A).

4 | DISCUSSION

LFNG encodes a glycosyltransferase (beta-1,3-*N*-acetylglucosaminyltransferase lunatic fringe) which is one of the most important regulators of the Notch pathway involved in somitogenesis. To date, 10 *LFNG* variants in seven SCD individuals have been reported (Table 1). Most of them (8 out of 10) are missense substitutions affecting the Fringe domain of the protein (aa 108–358) responsible for the *N*-acetylglucosamine transferase activity. We reported five unpublished *LFNG* variants: three missense substitutions affecting the Fringe domain (p.Arg174Leu, p.Val297Gly, and p.Asp355Asn); a nonsense variant (p.Asp289*) located one amino acid upstream of the active site (aa 290); and an intronic variant in the polypyrimidine tract of the *LFNG* exon 6 (c.822-5C>T). This latter, representing the first non-exonic variant in *LFNG* associated with SCD, did not lead to an aberrant splicing product but impacted on *LFNG* expression. Bioinformatic analysis of the mutant sequence predicted the formation of two binding sites for Celf1. Surprisingly, Celf1 has been reported to negatively regulate the expression of *dmrt2a/terra* by promoting mRNA decay in zebrafish, resulting in asymmetric somitogenesis and laterality defects.[12] Furthermore, homozygous start-loss variant (NM_006557.6:c.1A>Tp.[Met1?]) in *DMRT2* (MIM *604935—*dmrt2a/terra* human homolog gene) has been associated with SCD-like phenotype with severe ribs and vertebral malformations.[13]

Beside its peculiar expression in the skeleton, *LFNG* is also highly expressed in the ventricular zone of the neuroepithelium and inner ear structures. In zebrafish, *lfng* knockdown leads to a reduction of the brain size, whereas its overexpression is associated with an

increased number of neurons and large head circumference.[14] In line with these findings, a de novo heterozygous 380-kb duplication encompassing *LFNG* was identified in a subject with Asperger syndrome and macrocephaly.[15] On the other hand, all bi-allelic *LFNG* variants described in SCD individuals exert bona fide a loss-of-function (LoF) effect, being nonsense/frameshift variants or missense changes affecting the critical Fringe domain (Figure 1F). Therefore, it could be speculated that heterozygous LoF variants of *LFNG* might be tolerated, whereas bi-allelic LoF or monoallelic GoF might be pathogenic in humans. As aforementioned, *LFNG*, together with *MFNG* and other Notch pathway modifiers, plays a crucial role in the differentiation of inner hair cells.[16] Interestingly, proband 2 showed hearing impairment as another previously reported SCDO3 subject[7] (Table 1), thus potentially expanding the phenotypic spectrum associated with *LFNG* variants, although further observations are needed.

As observed in our probands, respiratory insufficiency is one of the most severe secondary complications of SCD, accounting for up to 44% of the mortality rate in the first 6 months of age,[17] and, thus, requires prompt medical management in neonates, including surgical treatment in selected cases.[18]

This study adds to previous evidence demonstrating the clinical usefulness of prenatal and neonatal trio-ES in the diagnosis of skeletal malformations, which is often challenging, especially in the prenatal setting.[19] Recent studies demonstrated that prenatal exome sequencing improves diagnostic yield in cases of fetal structural malformations.[20] In particular, two large prospective cohort studies reported a diagnostic rate of 8%–10% for trio-ES, which increased to 15%–24% in fetuses with skeletal phenotypes.[21,22] Exome sequencing has also been recognized as an effective diagnostic strategy for critically ill infants admitted to the NICU with suspected monogenic conditions, including skeletal disorders.[23] As stated by the ACMG, fetal-parental trio analysis is preferable to singleton (fetus-only) ES because assessment of trios allows enrichment of de novo variants, determination of phase for compound heterozygous variants, and confirmation of carrier status in both parents when a homozygous variant is detected.[24] In agreement with our findings, data filtering by phenotype-specific virtual gene panels allows a rapid and efficient interpretation of candidate variants and a better pregnancy and newborn management.

AUTHOR CONTRIBUTIONS

Mauro Lecca: Data curation, investigation, validation, visualization, writing—original draft. **Maria Francesca Bedeschi, Claudia Izzi, Chiara Dordoni, Berardo Rinaldi, Francesca Peluso, Stefano Giuseppe Caraffi, Federico Prefumo, Marino Signorelli, Matteo Zanzucchi, Silvia Bione, Claudia Ghigna, Silvia Sassi, Antonio Novelli:** Investigation. **Enza Maria Valente, Andrea Superti-Furga, Livia Garavelli:** Supervision, writing—review and editing. **Edoardo Errichiello:** Conceptualization, data curation, formal analysis, investigation, supervision, validation, visualization, writing—original draft, review and editing.

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CONFLICT OF INTEREST STATEMENT

The authors declare no conflict of interest.

PEER REVIEW

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DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

ETHICS STATEMENT

Written informed consent for publication was obtained from the proband's parents.

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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