



Identification of a homozygous frameshift variant in *RFLNA* in a patient with a typical phenotype of spondylocarpotarsal synostosis syndrome

Hitomi Shimizu^{1,2} · Satoshi Watanabe¹ · Akira Kinoshita² · Hiroyuki Mishima² · Gen Nishimura³ · Hiroyuki Moriuchi¹ · Koh-ichiro Yoshiura² · Sumito Dateki¹

Received: 7 December 2018 / Revised: 10 February 2019 / Accepted: 11 February 2019 / Published online: 22 February 2019
© The Author(s), under exclusive licence to The Japan Society of Human Genetics 2019

Abstract

Spondylocarpotarsal synostosis syndrome, a rare syndromic skeletal disorder characterized by disrupted vertebral segmentation with vertebral fusion, scoliosis, short stature, and carpal/tarsal synostosis, has been associated with biallelic truncating mutations in the filamin B gene or monoallelic mutations in the myosin heavy chain 3 gene. We herein report the case of a patient with a typical phenotype of spondylocarpotarsal synostosis syndrome who had a homozygous frameshift mutation in the refilin A gene (*RFLNA*) [c.241delC, p.(Leu81Cysfs*111)], which encodes one of the filamin-binding proteins. Refilins, filamins, and myosins play critical roles in forming perinuclear actin caps, which change the nuclear morphology during cell migration and differentiation. The present study implies that *RFLNA* is an additional causative gene for spondylocarpotarsal synostosis syndrome in humans and a defect in forming actin bundles and perinuclear actin caps may be a critical mechanism for the development of spondylocarpotarsal synostosis syndrome.

Introduction

Spondylocarpotarsal synostosis syndrome (SCT) (OMIM #272460) is characterized by disrupted vertebral segmentation with vertebral fusion, scoliosis, short stature, and carpal/tarsal synostosis. Mutations in filamin B (*FLNB*) (NM_001457) and myosin heavy chain 3 (*MYH3*) (NM_002470) have been identified in patients with autosomal recessive and autosomal dominant SCT, respectively [1–3].

Mutations in *FLNB* cause five distinct skeletal diseases (SCT, Larsen syndrome, atelosteogenesis type I, atelosteogenesis type III, and boomerang dysplasia). Among these, only SCT is inherited in an autosomal recessive manner; the

others are inherited in an autosomal dominant manner [4]. *FLNB* mutations have been reported in at least 16 families with SCT [5], all of whom showed either nonsense or frameshift biallelic mutations predicted to induce premature translation termination or consecutive changes in amino acid sequences, indicating that conditions brought about by severe *FLNB* defects are associated with phenotypes of SCT [1, 2, 4].

Filamins are dimeric actin-binding proteins [6]. Refilin A (*RFLNA*) and Refilin B (*RFLNB*) (also known as *FAM101A* and *FAM101B*, respectively) have been identified as vertebrate-specific short-lived filamin-binding proteins. Under TGF- β stimulation, filamins bind to *RFLNs* and transform their connecting actins into parallel bundle structures that accumulate each other to form perinuclear actin caps (Fig. 1a–c). A series of the processes above is important for cell migration and differentiation leading to endochondral ossification and skeletal development [6, 7].

We herein report the case of a Japanese boy with a typical phenotype of SCT who had a homozygous frameshift variant in *RFLNA* (NM_181709). We propose that *RFLNA* is an additional causative gene for SCT in humans.

✉ Sumito Dateki
sdateki1@nagasaki-u.ac.jp

¹ Department of Pediatrics, Nagasaki University Graduate School of Biomedical Sciences, Nagasaki, Japan

² Department of Human Genetics, Nagasaki University Graduate School of Biomedical Sciences, Nagasaki, Japan

³ Center for Intractable Disease, Saitama Medical University Hospital, Saitama, Japan

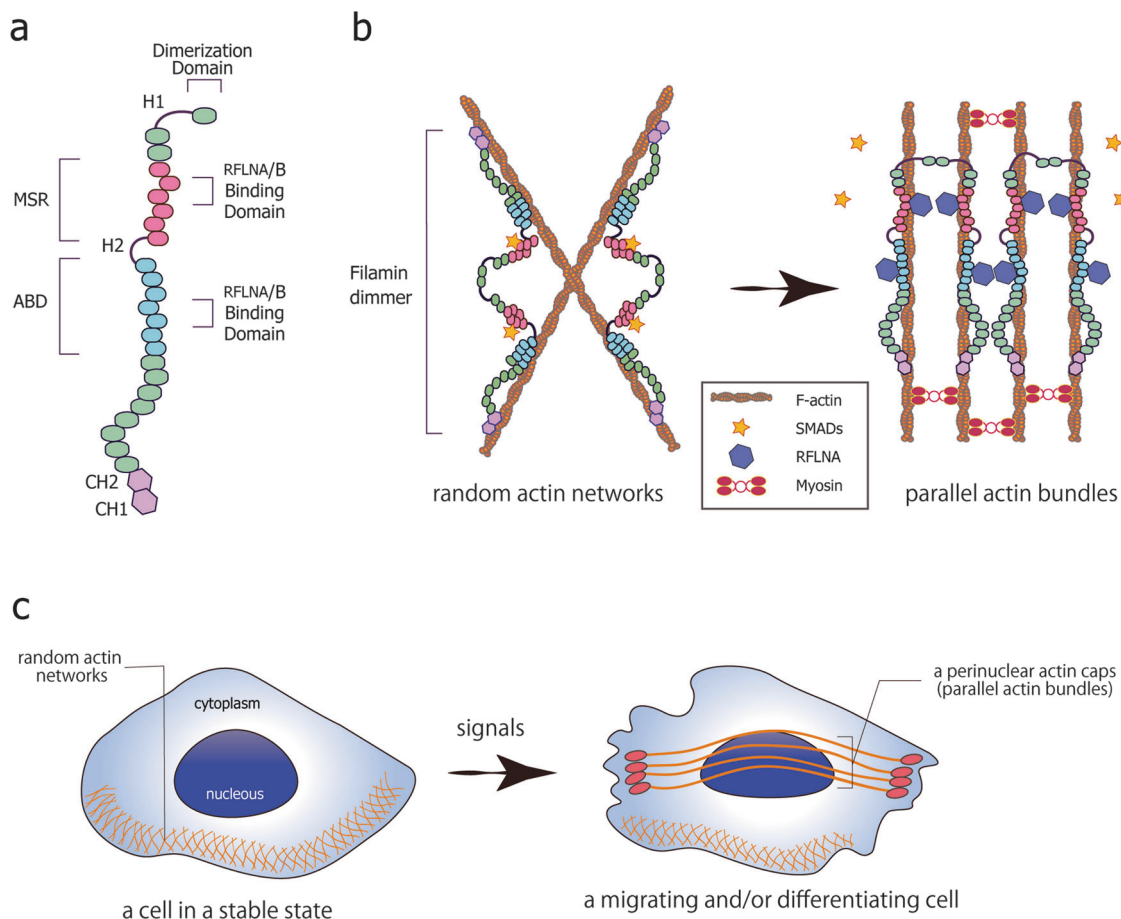


Fig. 1 A schematic illustration of filamins and the formation of parallel actin bundles and perinuclear actin caps. **a** The structure of a monomeric chain of filamins. Filamin contains two calponin homology domains (CH1 and CH2) that have actin-binding affinity followed by 24 β -pleated sheet immunoglobulin (Ig)-like repeats (ellipses). The repeats are interrupted by two flexible hinge regions (H1 and H2) that allow filamins for structural flexibility. The Ig-like repeats contain another actin-binding domain (ABD), two RFLNs binding domains, and a C-terminal domain that contains a mechanosensor region (MSR)

[5]. **b** Schematic illustration of a vertebrate filamin dimer (left) and formation of parallel actin bundles (right). Under the TGF- β stimulations, filamins bind to RFLNs and transform their connecting actin into a parallel bundle structure. During this process, MSRs release their holding mediators like SMADs to induce downstream signals. **c** The parallel actin bundles accumulate and produce perinuclear actin caps. These actin dynamics are necessary for cellular migration and differentiation. These figures are modified from those of Baudier et al.⁶ and Khatau et al.⁷

Materials and methods

Case report

The patient was born at 34 weeks of gestation. At birth, his length was 43 cm (-0.7 SD) and his weight was 2.35 kg ($+0.3$ SD). An X-ray examination at the time of birth showed seemingly normal segmented vertebrae. At 1 year and 2 months of age, the patient was referred to us because of severe short stature. His height was 67.2 cm (-3.7 SD), weight 7.8 kg (-2.2 SD), and occipital frontal circumference 47 cm ($+1.1$ SD). He also had mild facial dysmorphic features with frontal bossing and anteverted nares. A skeletal survey showed spondylar fusion mainly affecting the posterior neural arches and to a lesser degree the vertebral bodies with mild scoliosis and carpo-tarsal

synostosis (fusion of the capitate and hamate and probably that of the cuboid and lateral cuneiform) (Fig. 2). He was diagnosed with SCT based on his characteristic skeletal features, severe short stature, and progressive clinical course. At the last examination at 2 years and 3 months of age, he was 72.4 cm tall (-4.3 SD). His motor and mental development was normal. The patient's parents were non-consanguineous. The patient's father and elder brother were phenotypically normal, while his mother showed short stature (147 cm, -2.2 SD) without dysmorphic facial features or scoliosis.

Whole-exome sequencing

The family underwent trio whole-exome sequencing (WES). Genomic DNA extracted from peripheral blood

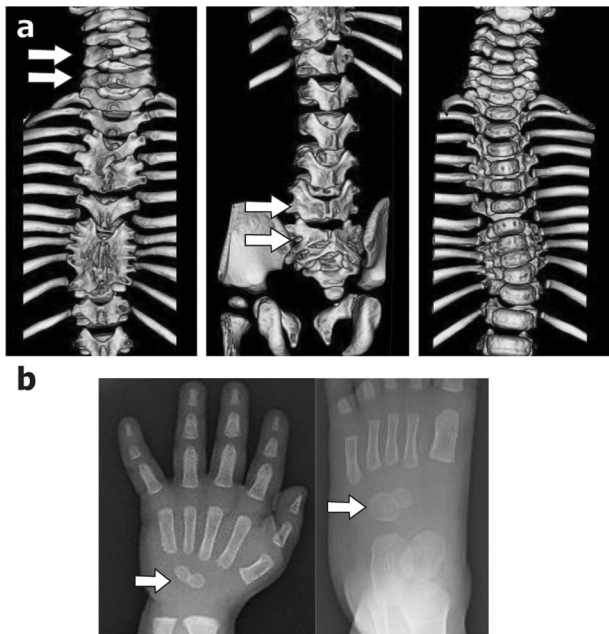


Fig. 2 Radiological examinations of the patient. **a** Dorsal (left, middle) and ventral (right) views of spinal three-dimensional computed tomography at 1 year 7 months of age show scoliosis, vertebral fusions and dysraphisms (white arrows). **b** Carpal (left) and tarsal (right) synostoses at 1 year 2 months of age (white arrows)

leukocytes was captured using Agilent SureSelect Exome Target Enrichment System v6 (Agilent Technologies, Santa Clara, CA, USA) and sequenced on a HiSeqTM 2500 (Illumina, San Diego, CA, USA) with 150 bp paired-end reads. Fastq format files were generated and aligned on the hg19/GRCh37 human reference genome sequence using the Novoalign software program (Novocraft Technologies, Kuala Lumpur, Malaysia). The Genome Analysis Toolkit (GATK HaplotypeCaller) was used for variant calling and consequently implemented in an in-house workflow management tool [8, 9]. Single nucleotide variations and insertions/deletions were annotated using the ANNOVAR software program [10]. Then, rare and deleterious variants were filtered using a previously described method [11]. Based on this pedigree, autosomal dominant, recessive, and X-linked recessive models of inheritance were assumed for the analysis. This study was approved by the Institutional Review Board Committee at Nagasaki University Graduate School of Biomedical Sciences.

PCR-based expression analyses of *RFLNA*

Total RNA was extracted from lymphoblastoid cell lines derived from the proband with the *RFLNA* mutation and the parents using the NucleoSpin RNA Plus kit (Takara, Shiga, Japan). RNA (2.0 µg) was reverse transcribed using the PrimeScriptTM II 1st strand cDNA Synthesis Kit (Takara). The obtained cDNA and control genome DNA were

amplified by PCR with primers for exon 2 (5'-GCATCAA GGTGAACCCGGA-3') and the 3' untranslated region in exon 3 (5'- GGCTGTTCTCTGCTTCAAGG-3') for the *RFLNA* gene, as well as those for exon 5 (5'- GAACAAGG TTAAAGCCGAGCC-3') and exon 6 (5'- GTGGCAGATT GACTCCTACCA-3') for the *PGKI* gene (NM_000291), which was utilized as an internal control. Subsequently, the PCR products were subjected to direct sequencing.

Results

Trio WES revealed a homozygous frameshift variant in the last exon 3 of the *RFLNA* gene in the patient (chr12:124 798 904C>- [GRCh37/hg19]; c.241delC [NM_181709]) (Fig. 3a). The parents were heterozygous for the variant. The mutational analyses were not done for the phenotypically normal elder brother. This variant is predicted to cause a frameshift at codon 81 for *RFLNA*, skip the initial 136th termination codon, and result in the production of an additional 110 aberrant amino acids (p.(Leu81Cysfs*111)) (NP_859060). PCR-based expression and sequence analyses using cDNA derived from lymphoblastoid cell lines showed that the mutant allele was expressed in the patient (Fig. 3b), and the mutant and the wild-type alleles were expressed in the parents with the heterozygous *RFLNA* variant (data not shown; Fig. 3b) [6]. The variant in *RFLNA* has not been registered in the following databases: 1000G (www.1000genomes.org), Exome Aggregation Consortium (ExAC; <http://exac.broadinstitute.org/>) and Integrative Japanese Genome Variation Database (3.5KJPN; <https://ijgvd.megabank.tohoku.ac.jp/>).

In addition, a rare heterozygous missense variant in the *FLNB* gene (chr3:58 121 852C>G [GRCh37/hg19]; c.4818C>G [NM_001457.3], p.Ile1606Met [NP_001448.2] [rs774972522]) was identified in the patient and the mother. The father had no deleterious variants in *FLNB*. The minor allele frequency of the c.4818C>G in *FLNB* variant in the general population was reported to be 0.27% in the 3.5 KJPN database. In silico analyses performed using PolyPhen-2 (<http://genetics.bwh.harvard.edu/pph2/>) and MutationTaster (<http://www.mutationtaster.org>) predicted that this rare variant would be pathogenic. The expression analyses of the proband revealed a biallelic expression of *FLNB* without abnormal splicing variants or exonic deletions (data not shown).

There were no mutations in *MYH3*, *RFLNB*, or other genes known to be related to vertebral segmentation formation [12].

Discussion

We identified a rare maternally derived missense *FLNB* variant (c.4818C>G, p.Ile1606Met) in the present patient

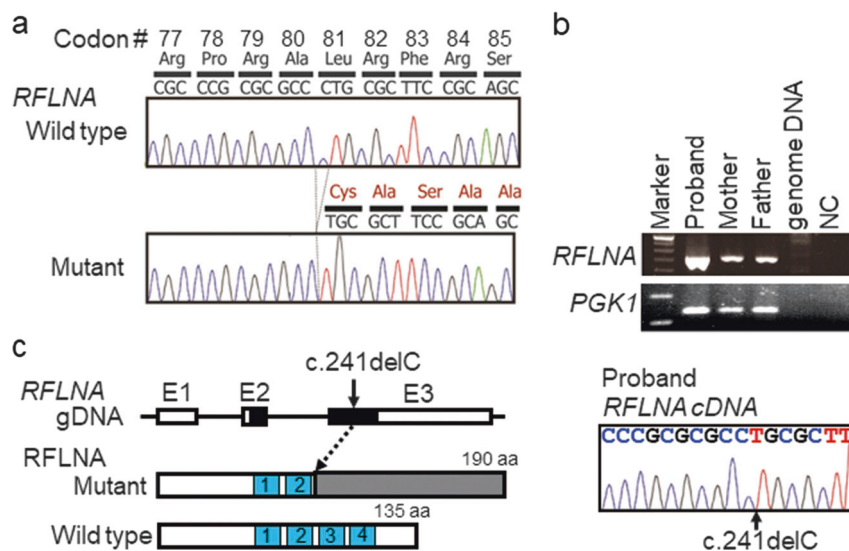


Fig. 3 The *RFLNA* variant of the proband. **a** Electrochromatograms delineating a homozygous frameshift *RFLNA* variant (c.241delC, p. (Leu81Cysfs*111)) (NM_181709, NP_859060.3) in the proband. **b** PCR-based expression analyses for *RFLNA* (35 cycles) (upper) and the sequencing analysis (lower). *PGK1* has been used as an internal control (20 cycles). The mutant *RFLNA* is expressed in lymphoblastoid cell lines derived from the proband as well as the parents with the

heterozygous *RFLNA* mutation. NC negative control. **c** The position of the *RFLNA* variant and the estimated structure of the mutant protein. This variant is predicted to skip the initial termination, and result in the production of an additional 110 aberrant amino acids (a gray box). This mutated protein is predicted to retain the filamin-binding domains (FBDs) 1 and 2 but lose the FBD3 and FBD4 (blue boxes)

with a typical phenotype of SCT. While SCT is caused by biallelic truncating mutations in *FLNB* [1, 2, 4], the expression analyses in this study showed a biallelic expression of *FLNB*, including normal transcripts of *FLNB* that originated from the paternal allele in the patient, indicating that the patient is certainly heterozygous for the *FLNB* variant. Furthermore, the *FLNB* variant has been identified among the general Japanese population. In addition, the mother with the same variant does not show the typical SCT phenotype. Collectively, the present data argue against any pathological role of the missense variant in the development of SCT or short stature remains tenable. Thus, a mutation(s) in a new, undiscovered gene(s) may be responsible for SCT in the patient.

In this regard, we identified a novel homozygous frameshift mutation in *RFLNA* in the patient, and propose the homozygous mutation of *RFLNA* as another genetic cause of SCT, based on the following findings. First, although mice with the single knockout of either *Rflna* or *Rflnb* (also known as *Cfm2* and *Cfm1*, respectively) displayed wild-type phenotypes, double knockout mice manifested progressive scoliosis, kyphosis, vertebral fusions, intervertebral disc defects, and growth retardation [13]. The above phenotype is similar to that of *Flnb*-deficient mice and of human SCT patients, indicating that defects of RFLN families may lead to the phenotype of SCT in humans [1, 2, 4]. At this point, there is a phenotypic difference between *Rflna* single knockout mice

and our patient with a homozygous *RFLNA* mutation. This may be associated with the difference of their genetic background and/or gene expression pattern [14]. Second, only a few heterozygous truncating variants and no homozygous null variants in *RFLNA* have been registered in ExAC database, implying that biallelic *RFLNA* mutations result in some pathogenic effects in humans. Third, *Rflna* is expressed in the vertebral primordia, vertebral bodies and carpal bones in embryonic mice and the expression is increased in prehypertrophic chondrocytes, implying the positive role of *RFLNA* in vertebral and carpal/tarsal bone development [15]. Fourth, a significantly decreased expression level of *RFLNA* has been observed in primary osteoblasts derived from the spinal vertebrae in patients with adolescent idiopathic scoliosis [16]. This result indicates that *RFLNA* has an important role in the normal development and growth of the vertebral column. Finally, the variant is predicted to retain the filamin-binding domains (FBDs) 1 and 2 but lose FBD3 and FBD4 (Fig. 3c) and thereby hardly form parallel actin bundles. Thus, the variant is likely a loss-of-function mutation, although the abnormal amino acid extension may result in the acquisition of some neomorphic functions. Indeed, primary rib chondrocytes from *Rflna* and *Rflnb* double knockout mice formed fewer actin bundles [13]. A biallelic *Flnb* defect is also predicted to affect the parallel actin bundle formation. In addition, *MYH3* mutations have been reported to alter TGF- β canonical signaling [3]. Thus, a defect in forming actin bundles and perinuclear actin caps may be a critical mechanism responsible for the development of SCT.

In conclusion, we propose, for the first time, an association between a homozygous mutation of *RFLNA* and SCT. Further studies and the accumulation of additional cases with *RFLNA* mutations are needed to clarify the pathogenic significance of *RFLNA* mutations.

Acknowledgements We thank the family who participated in this study. We also thank Yasuko Noguchi and Chisa Koga for their technical assistance. This work was supported by a grant for the Initiative on Rare and Undiagnosed Diseases in Pediatrics (no. 18gk0110012h0101) from the Japan Agency for Medical Research and Development (AMED), Tokyo, Japan.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Publisher's note: Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.

References

- Krakow D, Robertson SP, King LM, Morgan T, Sebald ET, Bertolotto C, et al. Mutations in the gene encoding filamin B disrupt vertebral segmentation, joint formation and skeletogenesis. *Nat Genet.* 2004;36:405–10.
- Farrington-Rock C, Kirilova V, Dillard-Telm L, Borowsky AD, Chalk S, Rock MJ, et al. Disruption of the *Flnb* gene in mice phenocopies the human disease spondylocarpotarsal synostosis syndrome. *Hum Mol Genet.* 2009;17:631–41.
- Zieba J, Zhang W, Chong JX, Forlenza KN, Martin JH, Heard K, et al. A postnatal role for embryonic myosin revealed by MYH3 mutations that alter TGF β signaling and cause autosomal dominant spondylocarpotarsal synostosis. *Sci Rep.* 2017;7:41803.
- Xu Q, Wu N, Cui L, Wu Z, Qiu G. Filamin B: the next hotspot in skeletal research? *J Genet Genom.* 2017;44:335–42.
- Salian S, Shukla A, Shah H, Bhat SN, Bhat VR, Nampoothiri S, et al. Seven additional families with spondylocarpotarsal synostosis syndrome with novel biallelic deleterious variants in *FLNB*. *Clin Genet.* 2018;94:159–64.
- Baudier J, Jenkins ZA, Robertson SP. The filamin-B-refilin axis - spatiotemporal regulators of the actin-cytoskeleton in development and disease. *J Cell Sci.* 2018;13:131.
- Khatau SB, Hale CM, Stewart-Hutchinson PJ, Patel MS, Stewart CL, Searson PC, et al. A perinuclear actin cap regulates nuclear shape. *Proc Natl Acad Sci Usa.* 2009;10:19017–22.
- McKenna A, Hanna M, Banks E, Sivachenko A, Cibulskis K, Kernytsky A, et al. The Genome Analysis Toolkit: a MapReduce framework for analyzing next-generation DNA sequencing data. *Genome Res.* 2010;20:1297–303.
- Mishima H, Sasaki K, Tanaka M, Tatebe O, Yoshiura K. Agile parallel bioinformatics workflow management using Pwraque. *BMC Res Notes.* 2011;4:331–8.
- Wang K, Li M, Hakonarson H. ANNOVAR: functional annotation of genetic variants from high-throughput sequencing data. *Nucleic Acids Res.* 2010;38:e164.
- Morimoto Y, Shimada-Sugimoto M, Otowa T, Yoshida S, Kinoshita A, Mishima H, et al. Whole-exome sequencing and gene-based rare variant association tests suggest that *PLA2G4E* might be a risk gene for panic disorder. *Transl Psychiatry.* 2018;8:41.
- Gibb S, Maroto M, Dale JK. The segmentation clock mechanism moves up a notch. *Trends Cell Biol.* 2010;20:593–600.
- Mizuhashi K, Kanamoto T, Moriishi T, Muranishi Y, Miyazaki T, Terada K, et al. Filamin-interacting proteins, *Cfm1* and *Cfm2*, are essential for the formation of cartilaginous skeletal elements. *Hum Mol Genet.* 2014;23:2953–67.
- Liao BY, Zhang J. Null mutations in human and mouse orthologs frequently result in different phenotypes. *Proc Natl Acad Sci USA.* 2008;105:6987–92.
- Gay O, Gilquin B, Nakamura F, Jenkins ZA, McCartney R, Krakow D, et al. RefilinB (*FAM101B*) targets filamin A to organize perinuclear actin networks and regulates nuclear shape. *Proc Natl Acad Sci USA.* 2011;12:11464–9.
- Fendri K, Patten SA, Kaufman GN, Zaouter C, Parent S, Grimard G, et al. Microarray expression profiling identifies genes with altered expression in Adolescent Idiopathic Scoliosis. *Eur Spine J.* 2013;22:1300–11.