A New Split Hand/Foot Malformation with Long Bone Deficiency Familial Case

Carmela Fusco^{1,*} Pasquelena De Nittis^{1,2,*} Ali Abdullah Alfaiz^{2,3} Maria Teresa Pellico¹
Bartolomeo Augello¹ Natascia Malerba¹ Leopoldo Zelante¹ Alexandre Reymond² Giuseppe Merla¹

¹ Medical Genetics Unit, IRCCS Casa Sollievo della Sofferenza, San Giovanni Rotondo (FG), Italy

² Center for Integrative Genomics, University of Lausanne, Lausanne, Switzerland

³ Bioinformatics Core Facility, Swiss Institute of Bioinformatics, Lausanne, Switzerland

Address for correspondence Giuseppe Merla, PhD, Medical Genetics Unit, IRCCS Casa Sollievo della Sofferenza, Poliambulatorio Giovanni Paolo II, I-71013 San Giovanni Rotondo (FG), Italy (e-mail: g.merla@operapadrepio.it).

J Pediatr Genet 2017;6:98–102.

Abstract

Keywords

- split-hand/foot malformation
- SHFLD3
- 17p13.3 microduplication
- osteogenic differentiation

Split hand/foot malformation with long bone deficiency (SHFLD) is a congenital limb anomaly where hands and/or feet cleft and syndactyly are associated with long bone defects, usually involving the tibia. Previously published data reported that 17p13.3 chromosomal duplication, including the *BHLHA9* gene, has been associated with the distinct entity, termed SHFLD3 (OMIM 612576), inherited as an autosomal dominant trait.

Here, we present a family with three members affected by SHFLD harboring *BHLHA9* duplication. We exploited in vitro differentiation system to promote proband's skin fibroblasts toward osteoblastic lineage, and we observed a slight but consistent delay in the mineralization pattern. This result possibly suggests an impairment of the osteogenic process in the affected members.

Introduction

Split hand/foot malformation with long bone deficiency (SHFLD) is a rare autosomal-dominant skeletal disorder with an estimated incidence of 1 per 1,000,000 live births. It is characterized by variable expressivity and incomplete penetrance. The phenotypic manifestations range from tibial defects, for example hemimelia, hypoplasia, aplasia or dysplasia, combined with severe median clefts of hands and feet, and missing or malformed central fingers.¹

The autosomal dominant SHFLD3 (OMIM [Online Mendelian Inheritance in Man] 612576) is associated with the 17p13.3 duplications/triplications whose breakpoints encompass the transcription factor basic helix-loop-helix family, member A9 (ID: 727857, *BHLHA9*).^{2–5} Moreover, among different reported clinical data, the deoxyribonucleic acid (DNA) copy number

involving *BHLHA9* constitutes a strong underlying factor with a dosage effect for the development of a range of limb malformations, with variable expressivity and incomplete penetrance.⁵

BHLHA9 encodes a putative basic helix-loop-helix DNA-binding protein. Knockdown of its ortholog in zebrafish (*bhlha9*) resulted in shortening of the pectoral fins,³ while a *Bhlha9* knockout mouse model exhibited various degrees of syndactyly in the forelimbs, with an incomplete separation of digits 2 and 3, the index and middle fingers.⁶ Mutational analysis on 17 patients affected by mesoaxial synostotic syndactyly with phalangeal reduction (OMIM 609432) revealed three neighboring homozygous missense mutations in the highly conserved DNA-binding region of the *BHLHA9* protein, confirming the role of the transcription factor in limb morphogenesis.⁷

We report a phenotypic and genotypic description of three SHFLD3 affected patients from the same family confirming, as reported in Petit et al^{1,8} and Klopocki et al,³ the autosomal pattern of inheritance of the disease associated

*These authors contributed equally to this work.

received

April 7, 2016

accepted after revision

July 19, 2016

published online

August 31, 2016

Copyright © 2017 by Georg Thieme Verlag KG, Stuttgart · New York

DOI <http://dx.doi.org/10.1055/s-0036-1588029>.
ISSN 2146-4596.

with 17p13.3 microduplication that includes *BHLHA9*. We performed in vitro osteogenic differentiation to get new insights on the putative mineralization defect in this condition.

Materials and Methods

Patients

A family with three members affected by SHFLD3 was enrolled at the Medical Genetics Unit of our Institute after approval by the Local Ethics Committee. Informed consent was signed for biospecimen collection and storage. Clinical data were obtained through patients' referring physicians and through their medical records.

Cell Cultures and Osteogenic Differentiation of Skin Fibroblast Cell Lines

Primary skin fibroblasts were grown in minimum essential medium supplemented with 1% L-Glutamine (Lonza Group, Basel, Switzerland), 10% fetal bovine serum (FBS, Lonza), and antibiotics. Harvested skin fibroblasts from passage 3 to 5 were cultured in osteogenic differentiation medium (Human Mesenchymal Stem Cell Osteogenic Differentiation Medium, hMSC, Lonza) supplemented with 0.1 μ M of dexamethasone, 50 μ M of ascorbate-2-phosphate, 10 mM of β -glycerol phosphate, L-Glutamine, Pen/Strep, and Mesenchymal Cell Growth Supplements (Lonza Group, Basel, Switzerland). The cells were kept in osteogenic medium up to 4 weeks. Every 3 days, the medium was completely replaced with fresh osteogenic induction medium. Non-induced control fibroblasts were maintained with standard medium on the same schedule to monitor the eventual spontaneous formation of osteoblast-like cells.

Staining for Mineralization

The degree of osteogenic differentiation was verified as early as the first week of induction by morphological changes and, at the end of the induction period, by the mineralization of the extracellular matrix, characteristic of late osteogenic differentiation. Von Kossa staining, envisaging a precipitation reaction with the anionic portion of phosphates, carbonates, and other salts,⁹ was used to evaluate cells' mineralization.

Formalin fixed cells were treated with 5% silver nitrate solution (Sigma-Aldrich, United States) for 60 minutes with exposure to ultraviolet light and followed by incubation with 5% sodium thiosulfate (Sigma-Aldrich, United States) for 5 minutes. Finally, a neutral red solution (Sigma-Aldrich, United States) was used to counterstain the nuclei. Calcium phosphates and carbonates appeared as dark brown deposits.

The ImageJ software (<http://rsbweb.nih.gov/ij/>) was used to determine the total area of von Kossa-positive nodules, as reported in Lin et al.¹⁰

Digital pictures taken with Nikon Camera (Nikon Instruments Inc., Netherlands) were imported into ImageJ, and the number and the area of the stained spots based on pixel values relative to the selected regions (region of interest), were measured using a custom written macro.

Quantitative Real-Time Polymerase Chain Reaction

Specific oligo sequences for genomic quantitative polymerase chain reaction (qPCR) assays were designed using the Primer Express software¹¹ with default parameters and based on the UCSC GRCh38/hg18 assembly (see supplementary ►Table 1 available in the online version of this article). QPCR reactions and calculations were made as reported in Howald et al.¹² Briefly, the reactions were performed with the Power SYBR Green technology according to the manufacturer's instructions (Thermo Scientific, United States) and set up in 384-well plates using a 7900 Real-Time PCR System (Thermo Scientific, United States). Three control DNAs from healthy subjects were included in each run as calibrators. *Hs_SAMSN1* and *Hs_NCAM2* were used as reference genes for genomic amplification. The cycle threshold values (Ct values) were used to calculate the fold differences by the Ct method. Each assay was made in triplicates.

Results

Phenotype Reporting

We identified a nuclear family originating from Southern Italy, with three members affected by SHFLD3. Its pedigree is reported in ►Fig. 1A, whereas the main limb manifestations are listed in supplementary ►Table 2 available in the online version of this article.

I-2: At birth, the proband presented a bilateral congenital malformation of the upper limbs and a severe tibial hypoplasia (►Fig. 1B). Anteroposterior radiographs of the upper limbs revealed right oligodactyly characterized by four fingers, and left bilateral asymmetric ectrodactyly. Right lower limb appeared bowed and hypoplastic, with a short fibula protruding under the knee.

II-2: The second son of the proband presents, since birth, a bilateral asymmetric ectrodactyly with lack of the third finger on the left hand, and only two digits on the right hand. The long bone defect involves the right tibia, with a severe hypoplasia and a compromised fibula protruding (►Fig. 1C). Cardiac ultrasound scan showed a small atrial septal defect.

II-3: The third sibling was diagnosed with hypoplasia of the tibia as the main lower limb deformity (►Fig. 1D). Other relevant clinical signs were not observed.

Molecular Analysis

Mutation analysis for known molecular causes of Split hand/foot malformation,^{13–15} including *TP63*, *DLX5*, and *WNT10B* genes, revealed no sequence alterations in the affected family members. Additionally, CytoScan HD array (Affymetrix, Santa Clara, California, United States) reported no pathogenic copy number variants.

Therefore, to assess the status of *BHLHA9*, whose duplication was associated with SHFLD,^{2,3} we performed genomic qPCR. Primers targeting specifically *BHLHA9* showed that all affected members (I-2, II-2, and II-3) carried duplication of the region spanning the *BHLHA9* locus (►Fig. 2A and supplementary ►Table 1 available in the online version of this article).

Fig. 1 Clinical features of SHFLD3 patients. **(A)** Two generations pedigree of the Italian family. Filled black symbols indicate members affected by SHFLD3. **(B)** Representative images of the affected mother on the newborn period (7 days of birth) (on the left) and pictures of her hands (top) and her radiographs (bottom) (on the right). **(C, D)** Representative photos of the upper and lower limb anomalies of the affected siblings.

Osteogenic Differentiation

To address the commitment capability toward the osteoblastic lineage, we differentiated fibroblasts from the proband and a healthy and unrelated individual into mature osteoblasts. Osteogenic differentiation, which was evaluated by von Kossa histochemical staining for calcium deposition, was evident in both the patient (I-2) and control cell lines (► Fig. 2B). Calcium phosphate deposits are indeed indicative of the formation of mineralized matrix and successful osteogenic commitment.⁹ Interestingly, the visualized von Kossa-positive area on the healthy differentiated cell line (234,75 μm^2 , black bars) appeared greater than that on paired I-2 mature osteoblasts (30,13 μm^2 , gray bars) (► Fig. 2C), suggesting a possible impaired mineralization process in patient's cell line.

Discussion

We here describe a new family case of SHFLD3 characterized by duplication of *BHLHA9* on chromosome 17p13.3. The three affected members of the family bear severe malformations of the distal limbs, including split hand combined with tibial hypoplasia. Specifically and consistent with the variable expres-

sivity and incomplete penetrance in SHFLD3, patient I-2 presents a bilateral limb defect and hypoplasia of the right tibia, patient II-2 has a bilateral asymmetric ectrodactyly, hypoplasia of the right tibia and a congenital heart defect, whereas patient II-3 displays an isolated hypoplasia of the tibia.

Limb development is a highly coordinated and complex process of growth and apoptosis. Among different distal limb deformities, SHFLD3 has been associated with copy number variation of the 17p13.3 region that includes only the *BHLHA9* gene. *BHLHA9* plays a role in distal limb development in *bhlha9*-deficient mice; therefore, this strengthens the idea that a genomic microduplication in 17p13.3 gives rise to SHFLD3 in humans owing to extra copies of *bhlha9* per se.⁶

Furthermore, a role for *BHLHA9* in the regulation of survival and/or proliferation of the cells in the progress zone of the developing limb has been anticipated using a zebrafish model knocked down for *bhlha9*,³ which support its involvement in limb development; however, the underlying molecular pathway has not been investigated yet.

In this study, skin fibroblast cell lines from patient's and healthy individuals were committed toward osteoblastic lineage, a tissue relevant for the disease. Human dermal

Fig. 2 Molecular findings of SHFLD3 patients and in vitro osteogenic differentiation. **(A)** Relative DNA amount was measured by quantitative polymerase chain reaction in three controls and in all family members. Each assay was made in triplicates. **(B)** Osteogenic differentiation was revealed by the formation of extracellular mineralized matrix, as shown by von Kossa staining. Control (left), fibroblast cell line derived from a healthy subject; I-2 (right), fibroblast cell line derived from the proband (I-2); noninduced (top), fibroblast cell lines maintained with standard medium; induced (bottom), fibroblast cell lines cultured in osteogenic differentiation medium. For all acquisitions the $\times 10$ magnification was used. **(C)** Quantification of possible differences in osteogenic potential measured in control-derived compared with I-2-derived osteoblast precursors (black and gray bars, respectively) using ImageJ software. Von Kossa stained areas (μm^2) and number of counted spots, representing the calcium deposits, were used as parameters for the measurement (see text for details). The quantification was achieved from two independent experiments and the mean and standard deviation are shown.

skin-derived fibroblasts, indeed, exhibit in vitro mesenchymal features and can be specialized toward connective tissue phenotypes, such as mature osteoblasts.¹⁶ As a result, we demonstrated that fibroblast cells reveal differentiation potential when exposed to favorable factors. Interestingly, von Kossa mineralization staining unveiled reduced calcium deposition in patients versus control's cells.

In summary, based on the altered osteogenic promotion observed in I-2-derived cell line, in addition to the significant contributions of previous published data, we suggest that *BHLHA9* duplication in the SHFLD3 may contribute to altered osteogenesis, likely resulting in a delayed mineralization process, and its over dosage may contribute to skeletal features. Nonetheless, further investigations and overlap with additional cases are warranted for the understanding of the underlying molecular mechanism(s) of this congenital skeletal anomaly.

Conflict of Interest

None.

Acknowledgments

The authors thank the patients and their parents for their participation and the members of the Lausanne Genomic Technologies Facility for technical help. A. A. A. is recipient of a scholarship from the Saudi Arabian National Guard Health Affairs. This work was supported by grants from the Italian Ministry of Health (Ricerca Corrente to G. M.) and the Swiss National Science Foundation (31003A_160203 to A.R.). We are grateful to the Genomic and Genetic Disorder Biobank, member of the Telethon Network of Genetic Biobanks funded by Telethon Italy (project no. GTB12001G), for the specimens biobanking. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

References

- Petit F, Jourdain AS, Andrieux J, et al. Split hand/foot malformation with long-bone deficiency and *BHLHA9* duplication: report of 13 new families. *Clin Genet* 2014;85(5):464–469
- Armour CM, Bulman DE, Jarinova O, et al. 17p13.3 microduplications are associated with split-hand/foot malformation and long-bone deficiency (SHFLD). *Eur J Hum Genet* 2011;19(11):1144–1151
- Klopocki E, Lohan S, Doelken SC, et al. Duplications of *BHLHA9* are associated with ectrodactyly and tibia hemimelia inherited in non-Mendelian fashion. *J Med Genet* 2012;49(2):119–125
- Nagata E, Kano H, Kato F, et al. Japanese founder duplications/triplications involving *BHLHA9* are associated with split-hand/foot malformation with or without long bone deficiency and Gollop-Wolfgang complex. *Orphanet J Rare Dis* 2014;9:125. Doi: 10.1186/s13023-014-0125-5
- Nagata E, Haga N, Fujisawa Y, Fukami M, Nishimura G, Ogata T. Femoral-tibial-digital malformations in a boy with the Japanese founder triplication of *BHLHA9*. *Am J Med Genet A* 2015;167A(12):3226–3228
- Schatz O, Langer E, Ben-Arie N. Gene dosage of the transcription factor *Fingerin* (*bHLHA9*) affects digit development and links syndactyly to ectrodactyly. *Hum Mol Genet* 2014;23(20):5394–5401
- Malik S, Percin FE, Bornholdt D, et al. Mutations affecting the *BHLHA9* DNA-binding domain cause MSSD, mesoaxial synostotic syndactyly with phalangeal reduction, Malik-Percin type. *Am J Hum Genet* 2014;95(6):649–659
- Petit F, Andrieux J, Demeer B, et al. Split-hand/foot malformation with long-bone deficiency and *BHLHA9* duplication: two cases and expansion of the phenotype to radial agenesis. *Eur J Med Genet* 2013;56(2):88–92
- Bills CE, Eisenberg H, Pallante SL. Complexes of organic acids with calcium phosphate: the von Kossa stain as a clue to the composition of bone mineral. *Johns Hopkins Med J* 1971;128(4):194–207
- Lin HH, Hwang SM, Wu SJ, et al. The osteoblastogenesis potential of adipose mesenchymal stem cells in myeloma patients who had received intensive therapy. *PLoS ONE* 2014;9(4):e94395. Doi: 10.1371/journal.pone.0094395
- Rozen S, Skaletsky H. Primer3 on the WWW for general users and for biologist programmers. *Methods Mol Biol* 2000;132:365–386
- Howald C, Merla G, Digilio MC, et al. Two high throughput technologies to detect segmental aneuploidies identify new Williams-Beuren syndrome patients with atypical deletions. *J Med Genet* 2006;43(3):266–273

- 13 Ianakiev P, Kilpatrick MW, Toudjarska I, Basel D, Beighton P, Tsipouras P. Split-hand/split-foot malformation is caused by mutations in the p63 gene on 3q27. *Am J Hum Genet* 2000;67(1):59–66
- 14 Rinne T, Hamel B, van Bokhoven H, Brunner HG. Pattern of p63 mutations and their phenotypes—update. *Am J Med Genet A* 2006;140(13):1396–1406
- 15 Lo Iacono N, Mantero S, Chiarelli A, et al. Regulation of *Dlx5* and *Dlx6* gene expression by p63 is involved in EEC and SHFM congenital limb defects. *Development* 2008;135(7):1377–1388
- 16 Lorenz K, Sicker M, Schmelzer E, et al. Multilineage differentiation potential of human dermal skin-derived fibroblasts. *Exp Dermatol* 2008;17(11):925–932