Compound Heterozygous Frameshift Mutations in MESD Cause a Lethal Syndrome Suggestive of Osteogenesis Imperfecta Type XX

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ABSTRACT

Multiple genes are known to be associated with osteogenesis imperfecta (OI), a phenotypically and genetically heterogeneous bone disorder, marked predominantly by low bone mineral density and increased risk of fractures. Recently, mutations affecting MESD, which encodes for a chaperone required for trafficking of the low-density lipoprotein receptors LRP5 and LRP6 in the endoplasmic reticulum, were described to cause autosomal-recessive OI XX in homozygous children. In the present study, whole-exome sequencing of three stillbirths in one family was performed to evaluate the presence of a hereditary disorder. To further characterize the skeletal phenotype, fetal autopsy, bone histology, and quantitative backscattered electron imaging (qBEI) were performed, and the results were compared with those from an age-matched control with regular skeletal phenotype. In each of the affected individuals, compound heterozygous mutations in MESD exon 2 and exon 3 were detected. Based on the skeletal phenotype, which was characterized by multiple intrauterine fractures and severe skeletal deformity, OI XX was diagnosed in these individuals. Histological evaluation of MESD specimens revealed an impaired osseous development with an altered osteocyte morphology and reduced canalicular connectivity. Moreover, analysis of bone mineral density distribution by qBEI indicated an impaired and more heterogeneous matrix mineralization in individuals with MESD mutations than in controls. In contrast to the previously reported phenotypes of individuals with OI XX, the more severe phenotype in the present study is likely explained by a mutation in exon 2, located within the chaperone domain of MESD, that leads to a complete loss of function, which indicates the relevance of MESD in early skeletal development. © 2021 The Authors. Journal of Bone and Mineral Research published by Wiley Periodicals LLC on behalf of American Society for Bone and Mineral Research (ASBMR).

KEY WORDS: MESODERM DEVELOPMENT GENE (MESD); OSTEOGENESIS IMPERFECTA (OI); LRP5; LRP6; WNT SIGNALING

Introduction

Osteogenesis imperfecta (OI) comprises a heterogeneous group of monogenetic disorders with a broad phenotypical spectrum and is characterized by a decrease in bone mass, a deterioration of bone microarchitecture and connective tissues, and an increased risk of fractures,1 creating challenges for diagnosis and treatment.2 OI, which was historically categorized according to the phenotype and inheritance pattern,3 is currently classified based on the clinical and radiographic...
phenotype, as well as the genetic analysis.\textsuperscript{(2,4)} In addition to COL1A1 (MIM: 120150) and COL1A2 (MIM: 120160), which encode the alpha chain of type I collagen and harbor the majority (approximately 85\%) of OI-causing mutations, 18 additional genes are known to be associated with OI (MIM: 112264, 616215, 605497, 607063, 614757, 300294, 607783, 610339, 301865, 123841, 600943, 606633, 182120, 611357, 611236, and 164820).\textsuperscript{(2,5–7)} Depending on the respective functions of the affected genes, other symptoms in addition to deterioration of bone mass and structure may include blue sclerae, hearing loss (conduct or sensory), dentogenesis imperfecta, malocclusion, and scoliosis, among others. Accordingly, patients should be admitted to specialized centers for optimal diagnostics, genetic counseling, and treatment.

Skeletal homeostasis requires the coordinated activity of bone-resorbing osteoclasts and bone-forming osteoblasts. The mechanosensitive osteocyte network contributes to this process by orchestrating bone turnover and bone mineralization processes. The pivotal role of the different WNT signaling pathways in regulating skeletal homeostasis, especially osteoblast differentiation and subsequent bone formation,\textsuperscript{(8)} became apparent by the detection of mutations in LRPS, which encodes the low-density lipoprotein-receptor (LRP) 5\textsuperscript{(9,10).} For the correct binding of WNT ligands, a dual receptor consisting of frizzled (FZD) and LRP5 or LRP6 is required to inhibit the \(\beta\)-catenin degradation complex, allowing the translocation of \(\beta\)-catenin into the nucleus followed by the initiation of WNT target gene transcription.\textsuperscript{(11)} Genetic variants may cause high bone mass syndromes,\textsuperscript{(12,13)} early-onset osteoporosis (EOP),\textsuperscript{(14,15)} and additional defects in other organs besides the skeleton.\textsuperscript{(16,17)} Moreover, autosomal recessive OI XV (OMIM: 615220) is caused by mutations in WNT1\textsuperscript{(18–20)} revealing the extensive potential role of WNT signaling skeletal maturation.

The mesoderm development gene (MESD) encodes the protein MESD (low-density lipoprotein receptor chaperone MESD), which plays a central role during embryogenesis by acting predominantly as a chaperone for the low-density lipoprotein receptors LRPS and LRPS in the endoplasmic reticulum (ER).\textsuperscript{(21–24)} Specifically, MESD enables the correct folding of \(\beta\)-propeller/epidermal growth factor (EGF) modules as well as its trafficking to the Golgi, which are crucial steps for further maturation.\textsuperscript{(23,25)} Observations in mice revealed that MESD mutations disrupt embryonic polarity, as LRPS and LRPS are not correctly localized in the cell, leading to impaired Wnt signaling and insufficient differentiation of mesoderm.\textsuperscript{(22–24)} Complete loss of function in homozygous Mesd-deficient mice led to lethal failure of mesodermal development after the beginning of gastrulation.\textsuperscript{(26)} Additionally, MESD was also shown to cause the effects observed in Lrp6 hypomorphic mutant mice\textsuperscript{(27)} and antagonize ligand binding to LRPS and LRPS on the cell surface.\textsuperscript{(28,29)}

OI type XX (MIM: 618644) was first described in five young individuals who were each homozygous for mutations in the third and final exon of MESD\textsuperscript{(30)} and was characterized by an increased incidence of fractures and a progressively deforming phenotype. The detected mutations removed a highly conserved ER retention domain but were located after the chaperone activity domain, resulting in a reduced but not abolished function of MESD. Accordingly, LRPS and LRPS trafficking was reduced but not eliminated.

Here, we present three individuals (II.1, II.2, and II.3), harboring compound heterozygous frameshift mutations in MESD exons two (c.265delG [p.Ala89Hisfs*8]) and three (c.607_611del [p. Thr203Alafs*26]), causing lethal OI type XX. Although the mRNA transcribed from the c.607_611del allele was still detectable, c.265delG led to a complete decay of the mutated mRNA, thus eliminating the chaperone activity of MESD. This indicates that the presence of only one MESD allele expressing a protein with residual chaperone function is sufficient for early embryonic development but not sufficient for survival.

**Materials and Methods**

Study design

We included five individuals within this analysis. All available medical records, images, and specimens were collected before the detailed skeletal assessment. Each of the three stillbirths displayed multiple fractures of the long bones and ribs, indicating a hereditary bone disorder, such as OI type II or infantile hypophosphatasia. To evaluate the degree of alteration, we included a stillbirth of the same gestational age without signs of skeletal irregularities as a control specimen. Informed consent was obtained from the parents in line with the local ethics committee and the Declaration of Helsinki.

Genetic analysis

Before presentation at our clinic, analyses of genes known to be associated with the development of severe forms of OI had been performed, including COL1A1–A2, CRTAP (MIM: 610682), LEPRE1 (MIM: 610915), PP1B (MIM: 259440), OSX (MIM: 613849), P4HB (MIM: 112240), SERPINH1 (MIM: 613848), and ALPL (MIM: 241500); these were performed to rule out infantile hypophosphatasia, and the analyses revealed no genetic variants. Whole-exome sequencing (WES) was carried out in the three stillbirths (SureSelect Human All Exon V6, Agilent, Santa Clara, CA, USA) using genomic DNA either isolated from frozen or from paraffin-embedded fetal material. For sequencing, a HiSeq 4000 sequencing machine (Illumina, San Diego, CA, USA) was used. Data were analyzed by the software tools VarFish\textsuperscript{(30)} and MutationDistiller\textsuperscript{(31)}

The variants were prioritized using the following phenotype terms: increased susceptibility to fractures (HP:0002659), progressive bowing of long bones (HP:0006383), and osteopenia (HP:0000938). MESD was ranked among the top 10 variants. The pathogenicity of the prioritized variants was judged using MutationTaster.\textsuperscript{(32)}

To test for alternative splicing in the heterozygous parents, mRNA was isolated (PAXgene, PAXgene Blood RNA, Becton Dickinson GmbH, Heidelberg, Germany). After reverse transcription using the RevertAid H Minus First Strand cDNA Synthesis Kit (Life Technologies, Darmstadt, Germany), the variants were prioritized using the following phenotype terms: increased susceptibility to fractures (HP:0002659), progressive bowing of long bones (HP:0006383), and osteopenia (HP:0000938). MESD was ranked among the top 10 variants. The pathogenicity of the prioritized variants was judged using MutationTaster.\textsuperscript{(32)}

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the ΔΔCt method using QuantStudio Design & Analysis Software, and GAPDH served as an endogenous control.

Histological analysis

After undergoing fetal autopsy (including photographic documentation, X-ray, cytogenetic, and histological examination), available specimens (femur, calvaria, and spine) were fixed in neutral-buffered formalin for 24 hours and processed for both undecalcified (embedding in methyl methacrylate) and decalcified histology (decalcification in 20% EDTA followed by embedding in paraffin wax). Next, the embedded specimens were cut into 4-μm-thick serial sections by a Microtome rotation microtome (Techno-Med GmbH, Munich, Germany) and then stained with the toluidine blue, von Kossa, trichrome Goldner, Ploton silver precipitation, or hematoxylin and eosin (H&E) staining methods. Unstained sections were evaluated for the lamellar structure of type I collagen using circularly polarized light microscopy (BX63, Olympus, Hamburg, Germany). Cellular and histomorphometric parameters were evaluated using OsteoMeasure software (Osteometrics, Atlanta, GA, USA) and according to the nomenclature of Dempster and colleagues.33 The number of canaliculi per osteocyte lacuna (N.Ot.Ca/Ot.Lc) was quantified in toluidine blue–stained sections using OsteoMeasure software (Osteometrics). Osteoid volume per bone volume (OV/BV), the number of osteoblasts/osteoclasts per bone perimeter (N.Ob/B.Pm; N.Oc/B.Pm) and the eroded surface per bone surface (ER/BS) were measured. Osteocyte connectivity was determined as previously established,34 using Ploton silver–stained sections of the vertebral column and the number of canaliculi per osteocyte lacuna (N.Ot.Ca/Ot.Lc) was quantified using ImageJ software (ImageJ 1.52, National Institutes of Health, Bethesda, MD, USA).

Quantitative backscattered electron imaging (qBEI) and acid etching

To further assess the bone mineral density distribution (BMDD) of the specimens, quantitative backscattered electron imaging (qBEI) was performed using a scanning electron microscope (LEO 435 VP, LEO Electron Microscopy Ltd., Cambridge, UK) with a backscattered electron detector (Type 202, K.E. Developments Ltd., Cambridge, UK) as previously described (20 kV, 680 ± 1 pA at a constant working distance). The generated gray value images representing the mean calcium weight percentage of the scanned bone surface were evaluated using a custom-made MATLAB (MathWorks, Natick, MA, USA) script, and the mean calcium weight percentage (CaMean), the most frequent calcium weight percentage (CaPeak), and the mineralization heterogeneity (CaWidth) were determined. To exclude hypermineralized cartilage remnants, we employed a threshold-based manual exclusion to process the images using ImageJ before evaluation.

The acid etching procedure was performed as established in our previous studies.35,36 Namely, polished samples were submerged in 9% phosphoric acid for 40 seconds before incubation with 5% sodium hypochlorite for 5 minutes. Air-dried specimens were sputter coated with gold and placed in a scanning electron microscope utilizing the secondary mode of imaging.

Results

Multiple fractures and skeletal deformities in OI type XX

The nonconsanguineous healthy parents of German origin presented at our specialized outpatient clinic after four miscarriages that occurred between the eighth and ninth gestational weeks and three stillbirths (Fig. 1A). They also had a daughter and two sons who were both healthy and were not available for our study. The family history was negative, and the parents did not show an increased rate of fractures. All three stillbirths occurred after prenatal ultrasound diagnosis of a skeletal disorder within the second trimester (Fig. 1A; Table 1). The three stillbirths (II.1, II.2, and II.3) had similar dysmorphisms consisting of a triangular face and retrognathism (II.1, II.2), as well as micromelia, angulated long bones, and bilateral clubfoot deformity (Fig. 1B–D; Table 1). In addition to the bluish color of the sclerae due to scleral thinning, no structural eye abnormalities, such as vitreoretinopathy or pseudoglioma, were observed during the fetal autopsy and histopathological examination. The thorax was narrowed (II.1, II.2), resulting in severe lung hypoplasia and congestion of the venous inflow tract of the heart with consecutive hepatomegaly and fetal hydrops. Compared with reference values, the weight of the lung was reduced up to 80%.37 Multiple rib fractures were observed in all stillbirths during fetal autopsy (Table 1), as well as overtubulation of the long bones. The abdomen revealed no irregularities, except for a double kidney in one individual (II.1). Brain development was age-appropriate in all cases.

WES revealing compound heterozygous frameshift mutations in MESD

Whole-exome sequencing of all three stillbirths revealed compound heterozygous frameshift variants affecting MESD exon 2 (c.265del [p.Ala89Hisfs*8]) and exon 3 (c.607_611del [p.Thr203Alafs*26]), each classified as American College of Medical Genetics (ACMG) class V (Fig. 2A, B). Whereas the variant in exon 2 is novel and the first described variant outside exon 3, the variant in exon 3 was already described by Moos and colleagues.60 The latter is located in the ER retention region of MESD, and residual chaperone function was shown for the resulting truncated protein.60 Both disease-causing variants were detected in the parents in a heterozygous state. RT-PCR analysis of mRNA isolated from the parents’ leukocytes revealed no evidence of alternative splicing (Supplemental Fig. S1). However, sequencing of the amplicons indicated a complete loss of the mRNA harboring the mutation c.265del, while the mRNA carrying the variant c.607_611del was readily detected (Supplemental Fig. S1).

Histological analysis showing irregular fracture repair and uncoupled bone cell function

The fetal autopsies (II.1, II.2, and II.3) revealed multiple intrauterine fractures of the shortened long bones and ribs as well as hypomineralized calvaria or even caput membranaceum (Fig. 3; Table 1). A detailed histological analysis was performed in the femur, calvaria, and spine (Fig. 4). The femoral bone (Fig. 4A) showed a linear fracture of the cortical and subchondral bone within the lower portion of the remodeling growth plate (Fig. 4B). The cortical bone in the area of the fracture was completely replaced by fibrous tissue. The fracture line within the remodeled mineralized cartilage tissue showed cystic spaces filled with bone and mineralized cartilage fragments as well as fibrous exudate. Although osteoclastic resorption activity is physiologically increased at this developmental stage, atypical osteoclast giant cells were noted. Both the bone cells proximal and distal to the fracture line appeared viable. The remaining
cortical bone and the calvaria consisted of woven bone with high numbers of enlarged osteocytes, showing irregular and thickened intercellular canaliculi (Fig. 4C, D). Evaluation of the vertebral specimen at low power revealed a regular structure of the ossification center (Fig. 4E). At higher power, enlarged osteoclasts were found within the growth plate area of

Fig 1. Family with several pregnancy losses and three stillbirths with similar phenotypes. (A) Pedigree of the family. The MESD heterozygous parents had a regular skeletal phenotype, whereas the three stillbirths displayed severe deformities and multiple intrauterine fractures. Four miscarriages before the first stillbirth are not shown. (B) Severe skeletal deformity of the long bones, narrowed thorax, and a triangular face were apparent. (C) Retrognathism and bilateral clubfoot deformity were observed. (D) Multiple rib fractures (white arrow) and lung hypoplasia secondary to narrowed thorax and pleural effusion were detected. Representative images B–D show individual II.1.
remodeled mineralized cartilage (Fig. 4F) and newly formed bone showed a superficial linear surface covered by a single layer of activated osteoblasts (Fig. 4G). An increased number of osteoclasts was observed in the MESD specimen (Fig. 4H), which also showed an increase in eroded surface compared with that in the control specimen, although no increase was observed in osteoclasts compared with the control (Fig. 4H). Within the comparably increased osteoids, enlarged preosteocytes were found. Deeper, newly formed bone within the resorption lacunae of the mineralized cartilage showed enlarged osteocytes and several thickened canaliculi, whereas the extracellular matrix appeared somewhat irregularly mineralized. When the specimens were investigated under polarized light, a highly irregular nonlamellar collagen orientation was apparent in the MESD specimen (Fig. 4I, J).

Impaired bone maturation and altered osteocyte morphology

To further evaluate the bone mineralization pattern, we performed qBEI analysis. In the spine, in addition to a difference in the size of the vertebrae, an irregularly shaped trabecular surface was visible, indicating increased resorption or irregular mineralization of the surface despite the presence of larger amounts of cartilage remnants (Fig. 5A). Additionally, after eliminating these cartilage remnants, the areas of the newly formed bone matrix were lower and more heterogeneously mineralized, resulting in lower CaMean and higher CaWidth, respectively, for the MESD specimen (Fig. 5B, C). To further investigate the osteocyte and canalicular network morphology, we performed acid etching, and the results revealed marked differences in the number of canaliculi per osteocyte among the different specimens (Fig. 5D). Furthermore, the number of canaliculi per osteocyte lacunae was significantly reduced in the MESD sample compared with the control specimen in the Ploton silver–stained sections, and the osteocytes appeared less mature, which together indicated impaired development of osteocyte morphology and the lacunocanalicular network.

Table 1. Clinical Characteristics of the Three Individuals With Compound Heterozygous MESSD Mutations

|                        | Individual 1         | Individual 2         | Individual 3         |
|------------------------|----------------------|----------------------|----------------------|
| Gestational age (weeks)| 26 + 0               | 22 + 5               | 14 + 5               |
| Crown-rump length (cm) | 25.0                 | 19.5                 | 9.2                  |
| Weight (g)             | 940                  | 502                  | 51.5                 |
| Generalized edema/ascites| Yes                 | Yes                  | Yes                  |
| Affection of organs    |                      |                      |                      |
| Brain                  | Age-appropriate      | Age-appropriate      | Age-appropriate      |
| Sclera                 | Blue                 | Blue                 | NA                   |
| Heart                  | Hypertrophic         | Age-appropriate      | Age-appropriate      |
| Lung                   | Hypoplastic          | Age-appropriate      | Age-appropriate      |
| Abdomen                | Double kidney        |                      |                      |
| Skeletal characteristics|                     |                      |                      |
| Skull/face             | Low mineralized skull| Low mineralized skull| Age-appropriate skull|
|                        | Triangular face      | Triangular face      | Triangular face      |
| Thorax deformity       | Narrowed             | Narrowed             | No                   |
|                        | Multiple rib fractures| Multiple rib fractures|                       |
| Extremities            | Micromelia, angulated clubfoot | Micromelia, angulated clubfoot | Micromelia, angulated clubfoot |
| Vertebral fractures    | No                   | No                   | No                   |
| Peripheral fractures   | Multiple             | Multiple             | Multiple             |
| Histology              | Wide growth plates in relation to very thin diaphyses | Wide growth plates in relation to very thin diaphyses | Calciﬁed trabeculae Highly irregular cartilage |
|                        | Thinned trabeculae  | Thinned trabeculae  |                       |
|                        | Impaired osteoid cartilage: less collagen | Loss of corticalis |                       |
|                        |                      | Only woven bone      | Mostly regular cartilage |

NA = not available.
detectable in mRNA derived from leukocytes from the carrier parent, indicating complete nonsense-mediated decay. Interestingly, the mutation c.607_611del (p.Thr203Alafs*26) in exon 3 downstream of the chaperone domain was previously described(6) in a child who died at the age of 7 months with a homozygous state, characterized by bilateral prenatal fractures of the humeri, ribs, femora, and clavicle. It was shown that loss-of-function variants in exon 3 escape nonsense-mediated decay and lead to the production of truncated MESD protein variants that retain residual chaperone function, although the ER retention domain encoded by exon 3 is no longer functional. Therefore, the lethal phenotype described here is due to a complete functional loss of one MESD allele, which cannot be compensated by the residual function of the second allele. As a consequence, the WNT signaling pathway might be substantially impaired,(40) leading to a severe and lethal effect on early mineralization and skeletal development processes. Moreover, these findings recapitulate the lethal effect observed in Mesd-deficient mice. This implies that very low MESD protein levels are sufficient for early development, while bone development beginning in the second trimester is dose sensitive as already shown for mutations in Lrp5 and Lrp6.(14)

Analysis of BMDD revealed inhomogeneous, impaired mineralization of newly formed bone in MESD individuals. Notably, the described changes indicating an increased osteoclast activity may be explained by the reported increase in osteoclastogenesis due to downregulation of OPG secretion from osteocytes(41) and differentiated osteoblasts(8) observed after deletion of Wnt/β-catenin signaling in vivo. Interestingly, increased quantities of cartilage remnants were observed, which has also been reported in a patient with OI type XII under antiresorptive treatment, leading to overall increased mineralization parameters,(42) pointing at disturbed development in terms of regular bone mineralization.(43) The observed altered osteocyte morphology and fractures of every long bone and concomitant numerous rib fractures. Remarkably, no fracture callus tissue was present, and irregularities in the newly formed bone and in the mineralized extracellular matrix were noted, indicating disturbed bone (re)modeling.

Blue sclerae were visible in the two older stillbirths. Moreover, the thoracic cavity was narrowed, with secondary restriction of the lung. However, we did not find any evidence of disturbed early embryonic development or patterning, which was found in Mesd-deficient mice. This implies that very low MESD protein levels are sufficient for early development, while bone development beginning in the second trimester is dose sensitive as already shown for mutations in Lrp5 and Lrp6.(14)

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reduced connectivity raise the question of the role of the osteocyte in disturbed bone development and remodeling, which remains to be elucidated. Although we can only speculate at this point, β-catenin activation in osteocytes has been shown to increase osteocyte differentiation in vivo (44) while inhibition of the Wnt signaling may impair osteocyte differentiation (45).

The combination of severe skeletal deformity, multiple fractures, irregular bone mineralization and microstructure, and blue sclerae supports characterizing this phenotype as OI and resembles the general clinical characteristics of OI (1, 6). The likelihood that the detected MESD variants cause OI type XX is also supported by previous reports pointing at the relevance of WNT signaling in skeletal development and bone mass regulation (6, 11, 18, 19). However, biallelic loss-of-function mutations in LRPS cause osteoporosis pseudoglioma (OPPG) syndrome (16) which represents a skeletal fragility syndrome (46) distinct from OI. Because BMDD data for OI is heterogenic (47–51) in general and comparability is limited here in particular, future studies elaborating on the presented findings are needed.
Fig 4. Assessment of the bone phenotype. (A) Whereas the angulating deformity of the femur was detectable at the macroscopic level (left image, II.1), impaired bone development was observed on microscopic level (right image, hematoxylin and eosin staining, II.1). (B) Magnification of the chronic pathological intrauterine metaphyseal fracture of the proximal femur. Remarkably, the fracture line was horizontal to the viable growth plate at the place of fractured and removed cortical bone, and cystic spaces at the bottom of the linear fracture were filled with fibrin and scattered bone fragments. The central cancellous bone was also viable. (C, D) Both the cortical femur (C) and the calvaria (D) skull bone tissues consisted of woven bone with enlarged numbers of osteocytes within irregular osteocyte lacunae. Several osteocyte lacunae were connected with broad intercellular osteocyte canaliculi (hematoxylin and eosin staining, individual II.1). (E) Overview of the ossification center of the vertebra. At low-power magnification, no substantial changes in the structure were found (toluidine blue staining, individual II.2). (F) Large osteoclasts (red arrow) with up to five isomorphic nuclei resorbing the mineralized cartilage of the growth plate were observed (toluidine blue staining, II.2). (G) Activated osteoblasts at the surface of primary spongiosa filled the irregular deep resorption lacunae of the mineralized cartilage with newly formed bone tissue. Enlarged osteocytes (white arrow) with irregular intercellular canaliculi embedded within mineralized extracellular matrix were found (toluidine blue staining, II.2). (H) Histomorphometric analysis of the control and MSED specimens (II.2) indicated increased osteoclast activity (n = 1 each). (I, J) Polarized light microscopy indicated a regularly oriented collagen structure in the control vertebrae (I) compared with the disoriented collagen structures in the MSED specimens (J), toluidine blue staining, II.2).
Fig 5. Evaluation of bone mineralization and osteocyte characteristics. (A) Backscattered electron microscopy overview of the vertebrae revealing differences in size and mineralization patterns between the control (left panel) and MESD specimens (right panel, II.2). At higher magnification, the increased amounts of cartilage remnants, the irregular trabecular surfaces, and the irregular mineralization of newly formed bone were observed. (B) BMDD histogram of control (blue line) and MESD indicating large amounts of hypermineralized cartilage remnants (CR) compared with mineralized bone (MB), resulting in overall higher mineralization in the MESD specimen. (C) After excluding cartilage remnants, lower and less homogenous mineralization of the bone matrix was observed in the MESD specimens than in the control, as indicated by lower CaMean and higher CaWidth, respectively. (D) Acid etching was performed, and the results indicated altered osteocyte morphology and reduced canaliculi in the MESD specimens compared with the control specimen. (E) Histological evaluation revealed a reduced number of canaliculi per osteocyte lacunae in MESD specimens compared with that in the control specimen. BMDD = bone mineral density distribution; CaMean = mean calcium weight percentage; CaWidth = heterogeneity of the BMDD represented by the standard deviation; Cn.N/Ot.Lc = number of canaliculi per osteocyte lacuna.
In conclusion, the relevance of MESD mutations in embryonic bone development is supported by the multiscale characterization of the skeletal phenotype. In the present study, we highlight for the first time the pivotal role of the MESD chaperone region in a white family of unrelated parents in the lethal phenotype observed in three stillbirths, which each harbored compound heterozygous mutations in exons 2 and 3, respectively. Multiple intrauterine fractures were observed in the affected individuals, indicating inferior bone quality, which might be mediated by a reduced osteocyte function. Further analyses of the microstructural tissue properties are recommended to explore ultrastructural changes in bone.

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