A variant in *SMOC2*, affecting the interaction with COL9A1, causes autosomal-dominant multiple epiphyseal dysplasia

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May 5, 2020

Abstract

Multiple epiphyseal dysplasia (MED) is a mild osteochondrodysplasia characterized by mild to moderate short stature and early-onset osteoarthritis. In this study, we found a family with MED with no linkage to known pathogenic genes. Whole-exome sequencing revealed a missense mutation (c.1076T>G, p.Leu359Arg, NM_001166412.2) in SPARC-related modular calcium binding 2 (*SMOC2*). We generated a mouse model by knocking-in the Smoc2 mutation. Mutant mice showed short-limbed dwarfism, disorganized and hypocellular proliferative zones and expanded hypertrophic zones in tibial growth plates. Study of the interaction between MED proteins and SMOC2 showed that SMOC2 and its extracellular calcium-binding (EC) domain could interact with collagen type IX α-1 (COL9A1), however, mutant SMOC2 could not. Our data indicated that *SMOC2* mutation is responsible for the MED phenotype. The mutation in SMOC2 affected the interaction between SMOC2 and COL9A1.

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Funding

This work was supported by the National Natural Science Foundation of China (81671114, 81471602,81741055,81873878) and Natural Science Foundation of Shandong Province (2015GSF118050, ZR2015HZ002, ZR2016HZ01, ZR2012HQ015) and the Key Technology Research and Development Program of Shandong (2016ZDJS07A08, 2018CXGC1211).

Abstract: Multiple epiphyseal dysplasia (MED) is a mild osteochondrodysplasia characterized by mild to moderate short stature and early-onset osteoarthritis. In this study, we found a family with MED with no linkage to known pathogenic genes. Whole-exome sequencing revealed a missense mutation (c.1076T>G, p.Leu359Arg, NM_001166412.2) in SPARC-related modular calcium binding 2 (*SMOC2*). We generated a mouse model by knocking-in the *Smoc2* mutation. Mutant mice showed short-limbed dwarfism, disorganized and hypocellular proliferative zones and expanded hypertrophic zones in tibial growth plates. Study of the interaction between MED proteins and SMOC2 showed that SMOC2 and its extracellular calcium-binding (EC) domain could interact with collagen type IX α-1 (COL9A1), however, mutant SMOC2 could not. Our data indicated that*SMOC2* mutation is responsible for the MED phenotype. The mutation in SMOC2 affected the interaction between SMOC2 and COL9A1.

KEYWORDS : Multiple epiphyseal dysplasia (MED); SPARC-related modular calcium binding 2 (SMOC2); Growth plate; Collagen type IX α-1 (COL9A1)

1 | Introduction

Multiple epiphyseal dysplasia (MED, MIM 132400) is a genotypically and phenotypically heterogeneous skeletal dysplasia and chondrodysplasia that affects epiphysis of long bones (Anthony, Munk, Skakun, & Masini, 2015; Chapman, Briggs, & Mortier, 2003). MED is characterized by mild to moderate shortness of stature and early-onset arthritis. Eight disease genes, which may be inherited in an autosomal dominant or recessive pattern, have been identified. Autosomal dominant variants include collagen oligomeric matrix protein (*COMP*) (Briggs et al., 1995; Hecht et al., 1995), collagen type IX α-1 (*COL9A1*) (Czarny-Ratajczak et al., 2001), collagen type IX α-2 (*COL9A2*) (Muragaki et al., 1996), collagen type IX α-3 (*COL9A3*) (Paassilta et al., 1999), matrilin-3 (*MATN3*) (Chapman et al., 2001) and collagen type II α-1 (*COL2A1*) (Dasa et al., 2019). Autosomal recessive variants are the sulfate transporter gene (*SLC26A2*) and calcium-activated nucleotidase-1 (*CANT1*) (Balasubramanian et al., 2017; Superti-Furga et al., 1999). All proteins encoded by the known MED-associated genes are involved in maintaining the structural integrity of the cartilage extracellular matrix (ECM). All variants in these genes account for the molecular basis of about 70% of MED cases (Balasubramanian et al., 2017). However, a number of MED cases have no identifiable genetic mutation, and additional genetic etiology of MED remain to be identified.

SPARC-related modular calcium binding 2 (*SMOC2*) and *SMOC1* , its closest homolog, are members of the protein family BM-40 (also known as secreted protein acidic and rich in cysteines [SPARC] or osteonectin) (Vannahme et al., 2003; Vannahme et al., 2002). BM-40 is a prototypic collagen-binding matricellular protein that participates in regulating cell–matrix interactions, in particular influencing bone mineralization, wound repair and other biological functions. The BM-40 family of modular extracellular proteins is characterized by a follistatin-like (FS) domain as well as an extracellular calcium-binding (EC) domain with two EF-hand calcium-binding motifs. SMOC1 and SMOC2 share a common domain organization, containing one FS domain, one EC domain, two TY domains and one SMOC domain, a novel domain with no known homologs. Increasing studies have shown that both proteins interact with the receptor-mediated signaling of several growth factors and play diverse roles in physiological processes involving matrix assembly and extensive tissue remodelling.

In this study, we investigated a family with autosomal dominant MED, by linkage analysis, we excluded all known genes responsible for MED, then detected a heterozygous c.1076T>G (p. Leu359Arg) missense mutation in *SMOC2* using exome sequencing. To determine the in vivo pathophysiologic mechanism of SMOC2 mutation, we generated c.1076T>G knock-in mice. In contrast to wild-type mice, mutant mice showed a dysplastic tibial growth plate with disordered cells in the proliferative zones and expanded hypertrophic zones. Co-immunoprecipitation supported that mutant SMOC2 lacked the ability to interact with collagen

IX but retained the ability to interact with COMP and MATN3.

2 | Materials and Methods

2.1 | Patients

This study was approved by the medical ethics committees of Shandong University, China and followed the principles of the Declaration of Helsinki. Before the study initiation, written informed consent was obtained from participating individuals. We were contacted by a Chinese family that had several adults with short stature and osteoarthritis. All patients in this family received history and physical examination, but only III-14 and IV-9 received X-ray examination on the affected joints. Genomic DNA was extracted from peripheral venous blood by using standard protocols.

2.2 | Exome sequencing, variant filtration and mutation detection

We performed exome sequencing of DNA from patients II7, III14 and IV9 in this family by using SureSelect Human All Exon Kit (Agilent, Santa Clara, CA) to capture the exome and HiSeq2000 platform (Illumina, San Diego, CA) for sequencing. All variations were filtered by using dbSNP137, the 1000 Genomes Project, HapMap8 databases and ExAC. Sanger sequencing was used to confirm the mutation in *SMOC2* in this family. PCR involved using 40 ng genomic DNA and Easy Taq (Transgen Biotech, Beijing). The PCR products were sequenced by Biosune Biotechnology (Shanghai) and compared with the reference sequence in NCBI (*https://blast.ncbi.nlm.nih.gov*).

2.3 | Cell culture

The human embryonic kidney 293T (HEK 293T) cell line was purchased from the Cell Bank. High-glucose Dulbecco's modified Eagle medium (DMEM) and fetal bovine serum (FBS) were purchased from Gibco (Thermo Fisher Scientific). The cells were cultured in DMEM containing 10% FBS, 100 U/ml penicillin (A603460) and 100 μg/ml streptomycin (A100382-0050, Sangon Biotech) at 37 with 5% CO2.

2.4 | Cell transfection

Recombinant lentiviruses and stably transfected cell line were established as described in previous study(Ma et al., 2015). Stably transfected cells were seeded in a 100-mm^2 dish at 80% density. After maintaining in DMEM with 10% FBS overnight, 4–12 μg pCMV3-COMP-HA (HG10173-CY), pCMV3-MATN3-HA (HG11951-UT), or pCMV3-COL9A1-HA (HG12231-CY, all Sino Biological, China) were transfected into stably transfected cells by using 12 μl PEI (BMS1003-A, Thermo Fisher Scientific). At 48 h after transfection, cells were collected.

2.5 | Construction of the targeting vector and generation of chimeric mice

A targeting vector containing *SMOC2* exon10, 11 and 12 and the mutation allele flanked by a loxP site and a loxP-neo cassette was constructed, which was introduced into mouse embryonic stem (ES) cells. After removing the cassette, the targeting ES cells were injected into C57BL/ 6 (B6) blastocysts to generate chimeras (F0), which were mated with C57BL/6 mice to generate F1 heterozygous offspring. Generation of the mouse model was performed by Cyagen Biosciences (Guangzhou, China). The genotypes of mice were determined by Sanger sequencing. The sequence containing the mutation was amplified with primers (online supplementary material table S4). The PCR products were sequenced by Biosune Biotechnology (Shanghai) and compared with the reference sequence in NCBI.

2.6 | Analysis of the skeleton

The body weights of littermates were measured on days 30 and 63. The body length measurements were taken from X-ray radiographs by using X-RAD225 OptiMAX. The lengths of isolated femurs and tibias were measured by vernier caliper.

2.7 | Skeleton tissue paraffin section

Hind limbs from littermates at postnatal day 21 (P21), 63 (P63) were dissected. After fixation overnight in 4% paraformaldehyde and decalcification in 20% EDTA for 1 month, bone samples were embedded in paraffin wax and cut into 4 μm sections.

2.8 | Histological analysis

For immunohistochemistry, the slides of tissue samples were soaked in 3% H_2O_2 (ZLI-9311, ZSGB-BIO, China) for 15 min to quench endogenous peroxidase activity. An amount of 0.1% trypsin (A100458-0050, Diamond) was used to recover antigen and 20% goat serum (ab7481, Abcam) was used for blocking. The sections were incubated overnight at 4 with anti-PCNA (1:20, AB0051, Abways Technology), anti-Ihh (1:100, 13388-I-AP, Cell Signaling Technology) or anti-RUNX2 (1:100, AF5186, Affinity Biosciences) and 1 h with the secondary antibody at room temperature. Then, sections were developed by using 3,3-diaminobenzidine (DAB) (ZLI-9017, ZSGB-BIO). After dehydrating, clearing and mounting, the slices were photographed by microscopy (BX41, OLYMPUS, JPN).

For H&E staining (G1005-100, Servicebio Technology, Wuhan, China), the 4-μm paraffin-embedded sections of tibia were stained in 10% hematoxylin for 5 min and in 1% eosin for 1 min.

For TUNEL analysis, One Step TUNEL Apoptosis Assay Kit (C1090, Beyotime Biotechnology, Shanghai) was used and visualization was by laser-scanning confocal microscopy (BX51, OLYMPUS, Japan). Nuclei were stained with DAPI (ab104139, Abcam) and apoptotic cells were labeled with Cyanine 3 (Cy3).

2.9 | Duolink proximity ligation assay (DPLA)

HEK 293T cells stably transfected with *SMOC2* and transiently transfected with *COMP* (HG10173-CY), *MATN3* (HG11951-UT) or*COL9A1* (HG12231-CY, all Sino Biological, China) were seeded on the monolayers. After fixation and permeabilization, monolayers were incubated in blocking solution for 1 h at 37, then incubated with the primary antibodies and PLA probes (Duolink In Situ PLA probe anti-mouse PLUS and anti-rabbit MINUS antibody) for 1 h at 37, then ligation solution for 30 min at 37, then finally amplification solution for 100 min at 37 to amplify the signal and in detection solution (DUO92007-100RXN, Sigma-Aldrich) for 30 min at 37. Cell nuclei were stained with DAPI.

2.10 | Co-immunoprecipitation (Co-IP)

The proteins from stably transfected cells transiently transfected with*COMP* , *MATN3* or *COL9A1* were extracted by using IP lysis buffer with 1% protease inhibitor cocktail (K1007, ApexBio Technology) and ultrasonication. Extracts were mixed with antibody-coated Dynabeads (10004D, Invitrogen, Thermo Fisher Scientific) and incubated at 4 for 3 h. After 3 washes in washing buffer, immunoprecipitated proteins were eluted in 7.5 μl 2×SDS loading buffer by heating at 99 for 10 min for western blot analysis.

2.11 | Western blot

Total cell lysates were collected in RIPA lysis Buffer (P0013C, Beyotime Biotechnology) with 1% protease inhibitor cocktail (K1007, ApexBio Technology) and 1% phosphatase inhibitor (K1015, ApexBio Technology). An amount of 35 or 40 μg whole cell lysates were resolved by 10% SDS-PAGE and transferred onto PVDF membranes (Merck Millipore, Germany). After incubation in 5% non-fat milk for 1 h at room temperature, the membrane was incubated with the antibodies anti-HA (1:10000, 66006-2-Ig), anti-MYC (1:10000, 60003-2-Ig), anti-BAX (1:1000, 50599-2-Ig), anti-BCL-2 (1:1000, 26593-1-Ap) or anti-GAPDH (1:20000, 60004-1-Ig, Proteintech Group) overnight at 4 and horseradish peroxidase-conjugated secondary antibody for 1 h at room temperature. Signals were visualized by ECL blotting detection reagents (32209, Thermo) and exposed to X-ray films (SUPER RX-N-C, FUJIFILM).

2.12 | Real time PCR (qRT-PCR)

Cartilages from proximal tibias were dissected from wild-type and mutant mice. Then total RNA from cartilages was isolated by using TRIzol (Invitrogen) and cDNA was generated by using random primers.

Real time PCR was performed with the SYBR Green Real Time PCR Master Mix (CW0957, CWBIO, CHN) and primers (Table S4).

2.13 | Solid phase binding assay (SPB)

COL9A1 (TP318130, OriGene Technologies) was coated in the 96-well plates at 40 ng/well. Nonspecific binding sites were blocked with 30% BSA for 2 h at temperature. Wild-type or mutant SMOC2 EC domain (Bioss) was added to the wells at increasing doses (10, 20, 50, 100 and 200 ng/well) and incubated for 2 h at room temperature. Wells were incubated with anti-SMOC2 primary antibody (1:1000, bs-7506R, Bioss) overnight at 4 and horseradish peroxidase-conjugated secondary antibody for 1 h at room temperature. Then 100 μl TMB (C520026, EL-TMB Chromogenic Reagent kit, Sangon Biotech) was added to every well and plates were kept in the dark until a blue color was obtained. Then, 50 μl stop buffer (C520026, EL-TMB Chromogenic Reagent kit, Sangon Biotech) was added to stop the reaction and OD at 450 nm was measured.

2.14 | Statistical analysis

Unpaired two-tailed Student t test was used to analyze the results of the body length and weight of$Smoc2^{+/+}$, $Smoc2^{L359R/+}$ and$Smoc2^{L359R/L359R}$ mice at P30 and P63, the length of tibia and femur of $Smoc2^{+/+}$, $Smoc2^{L359R/+}$ and$Smoc2^{L359R/L359R}$ mice at P30 and P63, the width of growth plates and the ratio of hypertrophic zone to that of a growth plate of $Smoc2^{+/+}$, $Smoc2^{L359R/+}$ and$Smoc2^{L359R/L359R}$ mice at P21 and P63 and immunostaining analysis. $P<0.05$ was considered statistically significant.

3 | Results

3.1 | Pedigree and clinical findings of the family with MED phenotypes ,which caused by a c.1076T>G missense mutation in *SMOC2*

In the four successive generations of the family, 8 patients were mild short stature and arthropathy; the male/female ratio was close to 1, which suggested an autosomal dominant inheritance pattern (Figure 1a). All the other patients had a history of pain in joints and a shorter stature than average (Table S1). The proband IV-9 was a 4-year-old girl with knee pain. Clinical examination showed knee joints laxity and swelling and mild genu vara. The history examination showed she often got knees pain after exercise from 2 years old and her mother, uncles and grandfather had histories of arthropathy. Radiographs revealed irregular epiphyses which proceed homogeneous ossification ('glacier crevice' sign) (Figure 1b). II-7 complained of more than 50 years of pain and stiffness in knees and figures. Clinical examination showed swelling of knee joints and finger joints (Figure 1b). III-14 had pain in knees and difficulties in walking since 6 years old. Radiographs of the knees of III-14 revealed flattening of the knee epiphyses and irregular and downward patellas (Figure 1b). All the clinical and radiological findings of the patients were accord with the main features of MED. Although lacking of more detailed radiological findings of the patients, we preferred to diagnose the patients in this family as MED by the clinical and history examinations and radiographic evaluations.

In order to confirmed whether the patients in this family affected MED, we performed genetic analysis of the MED pathogenetic genes on this family (data not shown). The results showed no linkage between the MED pathogenetic genes and the MED phenotypes. To find the pathogenic gene which caused the MED phenotypes, we performed exome sequencing of DNA from patients II7, III14 and IV9. 9 variants were left after filtered with the inheritance model, bioinformatic data including Polyphen-2, SIFT, mutationtaster and ExAC data. *PABPC3* was not confirmed by Sanger sequencing in this family, and another 7 variants, except*SMOC2* , did not cosegregate with the phenotype in this family (Table S2). Only NM_001166412.2: c.1076T>G, p.Leu359Arg (L359R) mutation in exon 11 of *SMOC2* cosegregated in this family (Figure 1c, Figure S1a). Amino acid sequence alignment of SMOC2 in 12 species showed that the leucine at position 359 was highly conserved (Figure S1b).

3.2 | $Smoc2^{L359R/+}$ and$Smoc2^{L359R/L359R}$ mice developed short-limbed dwarfism

In order to study the in vivo pathophysiologic mechanism of *SMOC2*mutation, we generated c.1076T>G

knock-in mice (Figure S2a). However, normal Mendelian ratios were not observed in the offspring of all matings. Rare homozygous mutant mice were generated (Table S3). The genotypes of the offspring were confirmed by Sanger sequencing (Figure S2b). The wild-type mice were labeled $Smoc2^{+/+}$, heterozygous mutant *Smoc2* mice $Smoc2^{L359R/+}$, and homozygous mutant *Smoc2* mice $Smoc2^{L359R/L359R}$.

The gross skeletons of mutant mice were normal as compared with $Smoc2^{+/+}$ mice at birth (not shown). At postnatal day 30 (P30), obvious differences were observed. The body length of $Smoc2^{L359R/+}$ mice was 7.2% reduced and that of $Smoc2^{L359R/L359R}$ mice was 6.5% reduced as compared with $Smoc2^{+/+}$ mice; the weight of $Smoc2^{L359R/+}$ mice was 2.2% reduced and that of $Smoc2^{L359R/L359R}$ mice was 9.7% reduced as compared with $Smoc2^{+/+}$ mice (Figure 2a, c). At P63, the difference in body length among all the mice did not expand. However, the weights of $Smoc2^{L359R/+}$ and $Smoc2^{L359R/L359R}$ mice were 10.8% and 12.1% reduced as compared with $Smoc2^{+/+}$ mice (Figure S3a). At P30, the femur and tibia length of $Smoc2^{L359R/+}$ mice was approximately 7% and 5.7% reduced as compared with $Smoc2^{+/+}$ mice. The lengths of femur and tibia of $Smoc2^{L359R/L359R}$ mice were more than 17% and 15.5% shorter than those of $Smoc2^{+/+}$ mice (Figure 2a, d). At P63, the tibia lengths of $Smoc2^{L359R/+}$ and $Smoc2^{L359R/L359R}$ mice were 7% and 8.5% shorter than those of $Smoc2^{+/+}$ mice and the reduction in femur lengths was reduced to approximately 5% and 8.1% (Figure S3b).

3.3 | $Smoc2^{L359R/+}$ and $Smoc2^{L359R/L359R}$ mice had disorganized growth plate and abnormal chondrocytes

To determine the effect of mutant *Smoc2* on morphology of tibial growth plates, we used haematoxylin and eosin (H&E) staining of tibial growth plates of all mice at P21 and P63. All tibial growth plates of studied mice were clearly divided into resting, proliferative and hypertrophic zones. At P21, the widths of the growth plates from $Smoc2^{L359R/+}$ mice became wider because the hypertrophic zones were almost 30% longer than those of $Smoc2^{+/+}$ mice, but the resting and proliferative zones were still similar to those of $Smoc2^{+/+}$ mice (Figure 2b, e). However, at P63, the widths of the hypertrophic zones and ratios of width of hypertrophic zone to that of growth plates tended to be normal (Figure 2b, e). At P21, as compared with $Smoc2^{+/+}$ mice, for $Smoc2^{L359R/L359R}$ mice, proliferative zones were almost 29% shorter and hypertrophic zones were almost 18% wider (Figure 2b, e). At P63, the proportions increased to 46% and 67% (Figure 2b, e).

In the tibial growth plates of $Smoc2^{+/+}$ mice at P21 and P63, the chondrocytes in the resting, proliferative and hypertrophic zones were closely aligned, well organized and arranged along the long axis of the tibia (Figure 3a). The tibial growth plates of $Smoc2^{L359R/+}$ and $Smoc2^{L359R/L359R}$ mice developed progressively dysplastic growth plates from birth. The number of chondrocytes in the tibial growth plates were reduced in these mice than $Smoc2^{+/+}$ mice (Figure 3b, c, d) and they were disorganized and failed to arranged into a column, so hypocellular areas could be observed in the proliferative zones and hyaline cartilage of proximal tibia (Figure 3a).

3.4 | Growth plates from mutant mice showed chondrocyte apoptosis and hypertrophy spatially dysregulated

To determine the mechanism of the expansion of hypertrophic zones in mutant mice tibial growth plates, we determined the relative levels of proliferation at P21 by using immunohistochemistry with proliferating cell nuclear antigen (PCNA), a marker of proliferation. We counted the number of PCNA-positive cells as compared with hematoxylin-stained cells in proliferative and hypertrophic zones from tibial growth plates from all studied mice (Figure 4a). We found no significant differences in ratio of PCNA-positive cells in both proliferative and hypertrophic zones from the tibial growth plates. We used TUNEL assay to determine whether decreased apoptosis contributed to the expanded hypertrophic zones in mutant mice. As expected, mutant mice showed less TUNEL-positive chondrocytes in the hypertrophic zone as compared with $Smoc2^{+/+}$ mice (Figure 4b). On western blot analysis at P21, Bcl-2 expression was increased and Bax expression was reduced in chondrocytes of mutant mice, which showed the lower level of apoptosis in the mutant mice. To test chondrocyte differentiation, we used immunohistochemistry and real time PCR (qPCR) analysis of Ihh, Runx2 and Col10a1 levels. Ihh, Runx2 and Col10a1 levels were increased in $Smoc2^{L359R/+}$ mice (Figure

4c). On immunohistochemistry of cell hypertrophy at P21 (Figure 4c), the expression area of Ihh (area between the black lines) was wider in mutant mice than *Smoc2*$^{+/+}$ mice. The expressions of Runx2 and Col10a1were higher in mutant mice than*Smoc2*$^{+/+}$ mice, especially in proliferative and hypertrophic zones. The dysregulated apoptosis and hypertrophy might lead to the expanded hypertrophic zones in the tibial growth plates from mutant mice.

3.5 | Changed interaction between MED proteins and mutant SMOC2

In previous studies, COMP, MATN3 and collagen IX, which are pathogenic proteins of MED (MED proteins), could interact with each other and play an important role in the pathogenic process. To study whether SMOC2 could interact with MED proteins and affect the occurrence and development of MED, we analyzed the interaction between SMOC2 and MED proteins. On immunofluorescence analysis of SMOC2 and the MED proteins, SMOC2 and MED proteins co-localized at the cytoplasm and pericellular matrix (Figure S4). We also used DPLA to investigate the interaction between COMP, MATN3, COL9A1 and SMOC2. HEK293T cells transfected with*SMOC2* and *COMP, MATN3* or *COL9A1* showed strong positive signals in and out of the cytoplasm. Therefore, SMOC2 and COMP, MATN3, COL9A1 were in proximity (<40 nm apart) (Figure 5a). Considering that MED proteins are combined in the ECM, we examined the ability of wild-type and mutant SMOC2 to combine with COMP, MATN3 or COL9A1 by co-immunoprecipitation. Immunoblot analysis revealed interactions between wild-type SMOC2 and COMP, MATN3 or COL9A1 and interactions between mutant SMOC2 and COMP or MATN3 except COL9A1 (Figure 5b, c, d). Verification was performed by using the FTST, EC and mutant EC domains. COL9A1 could be detected in the precipitate immunoprecipitated by the FTST and EC domain but not by the mutant EC domain (Figure 5e). To confirm this result, we performed SPB. We detected dose-dependent binding of wild-type or mutant SMOC2 EC domain to COL9A1, but as compared with the wild-type SMOC2 EC domain, the mutant EC domain showed much weaker binding to COL9A1 (Figure 5f).

4 | Discussion

In this study, we demonstrated a four-generation family with complicated MED phenotypes and identified an unreported pathogenic gene,*SMOC2* , establishing a close relationship of *SMOC2* with MED phenotypes for the first time. The missense mutation NM_001166412.2: c.1076T>G was suggested to be the causative variant.

MED is a genotypically and phenotypically heterogeneous skeletal dysplasia and chondrodysplasia. There are distinct phenotypes in different types of MED. For example, COMP-MED has severe phenotypes: muscular hypotonia, pseudomyopathy, joint laxity and mild genu vara. Radiographic findings often show the distal femoral and proximal tibial epiphyses are small and the ossification of metaphysis does not proceed homogeneously from the centre to the periphery which leads to 'glacier crevice' sign within the epiphysis. The main features of COL9-MED are joints pain and stiffness. The knees often have significant abnormities, such as irregular epiphysis and genu varus or valgus. DTDST-MED is characterized by joints limitation, flat epiphyses and double-layer patella (Unger, Bonafe, & Superti-Furga, 2008). The proband IV-9 was a 4-year-old girl with knees pain mild genu vara. Radiographs revealed irregular epiphyses which was glacier crevice sign and the surface of epiphyses were saw-tooth. In addition, the history examination showed she often got knees pain after exercise from 2 years old and her mother, uncles and grandfather had histories of arthropathy, which was a typical MED history. All the clinical and radiographic findings and history examination were in accordance with MED phenotypes and history. Other patients also had short statures and histories of arthropathy. Although lacking of more detailed radiological findings in hip, shoulders, wrists, fingers and vertebra of the patients, we preferred to diagnose the patients in this family as MED by the clinical and radiographic findings and history examinations.

There were some similar phenotypes in the *mSmoc2* knock-in mouse to the patients in this family, such as short statures, short limbs and abnormalities in epiphyses, which were often found in MED patients and the MED mouse models in previous studies. The short-limbed dwarfism had been observed in a *Matn3* knock-in mouse model (Leighton et al., 2007) and a *Collagen IX* and *Comp* double knock-out mouse model

(Blumbach, Niehoff, Paulsson, & Zaucke, 2008). The enlarged hypertrophic zone in tibia growth plates in the *mSmoc2* knock-in mice model had been detected in *Matn3* knockout mice models (Leighton et al., 2007; van der Weyden et al., 2006). The meaningful phenotype, a disrupted proliferative zone with disorganized columns and some areas of hypocellularity in the tibial growth plate of*mSmoc2* knock-in mice model, was commonly found in the *COMP*(Posey et al., 2009), *MATN3* (Leighton et al., 2007; van der Weyden et al., 2006) or *COL9A1* (Blumbach et al., 2008; Dreier, Opolka, Grifka, Bruckner, & Grassel, 2008) knock-out or knock-in mouse models which were component of extracellular matrix and MED caused genes.

SMOC2 has been found involved in cell cycle progression by maintaining integrin-linked kinase activity during the G1 phase (Liu, Lu, Cardoso, & Vaziri, 2008). Both a homozygous mutation (c.84+1G>T) in the canonical-splice donor site of intron 1 and c.681T>A (p.C227X) nonsense mutations in *SMOC2*caused dental development defects (Mendoza-Londono et al., 2015; Sasaki, Hohenester, Gohring, & Timpl, 1998). However, no dental defects were observed in our family or the knock-in mouse model. Recent study has shown that overexpressing *SMOC2* in osteoprogenitor cells inhibits osteogenic differentiation and ECM mineralization (Peeters et al., 2018). Study of Xenopus and Drosophila showed that SMOC can activate MAPK signals, thereby inhibiting the BMP signaling downstream of its receptor (Thomas, Canelos, Luyten, & Moos, 2009; Thomas et al., 2017).

The extracellular matrix occupies the major volume fraction of the joint cartilage and is responsible for its main functions. These functions are engendered by two supramolecular systems, the collagen-containing fibrils and the extrafibrillar matrix. Cartilage fibrils contain at least collagens II and IX. The extrafibrillar matrix comprises mainly cartilage-specific proteoglycan and perifibrillar molecules which are neither collagens nor proteoglycans. As previous study has shown, COMP, MATN3, collagen IX and collagens II, which are known MED causative genes, bind directly or indirectly to the each other. Mutations of these genes may cause aberrant function or undesirable protein-protein interactions which lead to the MED. SMOC2 and its homologue SMOC1 are members of the BM-40/SPARC/Osteonectin family of structurally-related matricellular extracellular proteins. In this present, we detected colocalization in the ECM of growth plate and interaction between SMOC2 and COMP, MATN3 and collagen IX (Figure S4), which raise a question whether mutant SMOC2 alter these interaction to cause the MED.

SMOC2 and its homologue SMOC1 contain an FS domain, two TY domains separated by one SMOC domain, and an EC domain. The EC domain of BM-40 protein families have diverse biological functions such as binding to collagenous proteins (Sasaki et al., 1998). Homozygous missense variants in the EC domain of BM-40 abolished the affinity of BM-40 to collagen type I and caused recessive osteogenesis imperfecta type IV (Mendoza-Londono, 2015). Previous study has shown that the EC domain of SMOC2 is fully conserved and is presumably important by binding calcium for the structure of SMOC2 (Novinec, Kovacic, Skrlj, Turk, & Lenarcic, 2008; Vannahme et al., 2003). The SMOC2 EC domain mediates cell attachment by binding to αvβ6 and αvβ1 integrins on cell surface receptors (Maier, Paulsson, & Hartmann, 2008). In the present report, the Leu359Arg mutant in SMOC2 responsible for MED in our family is strictly conserved in evolution and is located in the first EF-hand motif of the EC domain. When we first focused on the interaction of SMOC2 and collagen IX, we found that the mutation could affect the interaction affinity of SMOC2 and collagen IX. So, the reduced interaction between SMOC2 and collagen IX may alter the biological function of collagen IX or SMOC2 and result in this MED. Certainly, its detailed mechanism needs to be elucidated.

In conclusion, our evidence supports that *SMOC2* is a new MED-causative gene and illustrates the importance of *SMOC2* in the development of cartilage and long bones. Ultimately, a complete understanding of the molecular genetics and cell-matrix pathophysiology of MED will aid in the diagnosis, prognosis and treatment of patients and families with MED and will also help in understanding the disease mechanisms of more common conditions such as osteoarthritis.

Acknowledgements

The authors are grateful to the patients and their families for their participation and the Linyi Peoples Hospital for put us in contact with the patients and their families.

conflict of interests

The authors declare no conflict of interest.

Data Availability

All data included in this study are available upon request by contact with the corresponding author.

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Figure 1 Identification of a mutation in *SMOC2* in a Chinese MED family. (a) Pedigree of a Chinese family with autosomal dominant MED phenotypes in this study. (b) Clinical photographs and radiographs of three affected individuals in this family. The pictures in the left column are clinical photographs, lateral view and anterior-posterior (AP) view of the swelling knee joints of IV9, which showed swelling joint and 'glacier crevice' sign in epiphysis. The pictures in the middle column are clinical photographs of II7, which showed the swelling knee joints and finger joints. The pictures in the right column are the lateral view and AP view of the knees of III14, which showed flattening of the knee epiphyses and downward patella. (c) Partial *SMOC2*sequence showed the heterozygous c.1076T>G, p.Leu359Arg mutation in exon 11 of the *SMOC2* gene in the affected family members compared to unaffected family members (normal). Mutant codon (CTG to CGG) is marked by a black box.

Figure 2 The development of mutant mice was inhibited. (a) Photographs and radiographs of $Smoc2^{+/+}$,$Smoc2^{L359R/+}$ and$Smoc2^{L359R/L359R}$ mice and femurs and tibias of them at P30. Black dotted lines are aligned at the tip of the nose and the top of the pelvis of the $Smoc2^{+/+}$ mouse. (c) Body lengths and weights of all mice were measured at P30. (n=8:8:2, P¡0.05, P¡0.01 by t-test.). (d) Lengths of femurs and tibias of all mice at P30. (n=5:5:2). (b) Hematoxylin and eosin (H&E) staining of tibial growth plates and the hypertrophic zone of tibial growth plate from P21 (the first row) and P63 (the second row) mice. RZ, resting zone; PZ, proliferative zone; HZ, hypertrophic zone; GP, growth plate. (e) Widths of growth plates, proliferative zones and hypertrophic zones of all mice at P21 (left) and P63 (right) (n=5:5:2).

Figure 3 Histological analysis of the tibial growth plate showing growth plate abnormalities. (a) The first row is H&E staining of the tibial growth plate of P21 mice showing a disrupted proliferative zone with disorganized columns (black box) and some areas of hypocellularity (black pentagram) in the tibial growth plate from$Smoc2^{L359R/+}$ and$Smoc2^{L359R/L359R}$ mice. The second and third rows are H&E staining showing a significantly decreasing cell density (black pentagram) and disorganized columns (black box) in the growth plate and hyaline cartilage (arrowheads) of proximal tibia from$Smoc2^{L359R/+}$ and$Smoc2^{L359R/L359R}$ mice compared to$Smoc2^{+/+}$ mice at P63. (b, c, d): Number of chondrocytes in tibial growth plates at P21 and P63 and number of chondrocytes in hyaline cartilage of proximal tibia at P63 (n=5:5:2, P¡0.05, P¡0.01, P¡0.001 by t-test).

Figure 4 Analysis of proliferation, apoptosis and hypertrophy of chondrocytes in tibial growth plate. (a) Immunohistochemistry of cell proliferation by anti-PCNA antibody on P21 mice. The relative proliferation was calculated by comparing the number of PCNA-positive chondrocytes to total number of chondrocytes in the proliferative or hypertrophic zone (n=5:5:2). (b) Apoptosis was measured in the tibia of P21 mice by TUNEL assay and anti-Bax and anti-Bcl-2 antibodies. The relative apoptosis was calculated by comparing the number of apoptotic chondrocytes to total number of chondrocytes in the hypertrophic zone. Nuclei were stained with DAPI (blue) and apoptotic cells were labeled by Cy3 (red) (n=5:5:2). (c) Immunohistochemistry of cell hypertrophy with anti-Ihh, anti-Runx2 and anti-Col10a1 antibodies in P21 mice. The expression area of Ihh (the area between the black lines) in mice. Real-time PCR of the cartilage from proximal tibia of mRNA expression of Ihh, Runx2 and Col10a1 (n=5:5:2) (P¡0.05,P¡0.001 by t-test).

Figure 5 Changed interaction between MED proteins and mutant SMOC2. (a) Duolink Proximity Ligation Assay showed SMOC2 could bind to COMP, MATN3 or COL9A1. The pictures in the top right corner are magnified images of the yellow boxes. The red dots represent that distances between SMOC2 and COMP, MATN3 or COL9A1 was < 40 nm. (b, c, d, e) Co-immunoprecipitation showed the interaction of SMOC2 and COMP, MATN3 or COL9A1. HA-tagged COMP and HA-tagged MATN3 could be detected in the precipitated immunoprecipitation by MYC-tagged wild-type SMOC2, MYC-tagged mutant SMOC2 or MYC-tagged mutant EC domain of SMOC2. HA-tagged COL9A1 could only be detected in the precipitated immunoprecipitation by MYC-tagged wild-type SMOC2. (f) Solid phase binding assay detects binding of SMOC2 EC domain or mutant SMOC2 EC domain to COL9A1. Increasing concentrations of SMOC2 EC domain or mutant SMOC2 EC domain were added to COL9A1-coated plates. Binding was revealed with anti-SMOC2 antibodies.