

Mutations in Frizzled Class Receptor 4 Associated With Congenital Cavus Foot Deformity

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Abstract

Background

Congenital cavus foot deformity (CFD) (congenital =present at birth) is a disease very disabling that could connected to the mobility, neurologic entities and the imbalance of synergistic intrinsic and extrinsic muscles of the patients, and the problem is even dynamic (often progressive). Here, we report the clinical and radiographic manifestations of one Chinese Han congenital CFD family and 23 congenital CFD patients. In the congenital CFD family, the proband's mother and brother are also CFD patients.

Results

We performed whole-exome sequencing for three patients and two healthy people in this family, and sequenced the Frizzled Class Receptor 4 (FZD4) for the other. One novel FZD4 mutation (exon2 c.1589G>A; p.G530E, NM_012193) was identified. Then mutations in FZD4 gene were further examined in 23 congenital CFD patients, and also find FZD4 mutation (FZD4:NM_012193:exon1:c.205C>T:p.H69Y) in one congenital CFD patient.

Conclusions

Our study suggested that the congenital cavus foot deformity might be associated with the identified mutations in FZD4.

Introduction

Cavus is the foot deformity described as a high plantar arch and a fixed forefoot equinus (1). Congenital cavus foot deformity (congenital =present at birth) is a disease very disabling that could connected to the mobility, neurologic entities and the imbalance of synergistic intrinsic and extrinsic muscles of the patients, and the problem is even dynamic (often progressive) (1-3). When the cavus foot has become a rigid posture, surgical options become more limited (3, 4). Early recognition of the deformity and appropriate treatment is important. Congenital cavus foot deformity is an inheritance disease, a lot of familial patients have been reported (5, 6), but no causative genes associated with it. Although there are no statistics for Congenital cavus foot deformity, the morbidity of clubfoot or congenital talipes equinovarus (CTEV) is with a worldwide incidence of approximately 1 in 1000 (7), which is a hindfoot deformity caused by malalignment at the talocalcaneonavicular complex. Previous study demonstrated that, cavus deformity could be progressive or caused by paralytic soft tissue diseases, osteoarticular diseases, or trauma (1). The presence of bilateral clubfeet increases the likelihood of underlying genetic factors (mostly monogenic diseases), which should employ diagnostic genetic testing.

Frizzled Class Receptor 4 (FZD4) is a member of the frizzled gene family. Members of this family encode seven-transmembrane domain proteins that are receptors for the Wingless type MMTV integration site

family of signaling proteins (8, 9). Most frizzled receptors are coupled to the beta-catenin (CTNNB1) canonical signaling pathway, which leads to the inhibition of GSK-3 kinase, nuclear accumulation of CTNNB1, and activation of disheveled proteins and Wnt target genes, (10, 11). On the one hand, Wnt/ β -catenin signaling has emerged as a central regulator of bone metabolism over the last two decades, with an influence on nearly all aspects of skeletal development biological process (12, 13). As the specific frizzled (Fzd) receptors that recognize Wnt ligands, FZD4 is required for normal bone development and mineralization, and highly expressed by the osteoblast and genetic disruption of Fzd4 expression in mature osteoblasts impaired in vivo mice (14, 15). On the other hand, according to the previous reports, FZD4 is responsible for autosomal dominant inheritance diseases, [Exudative Vitreoretinopathy 1](#) and [Exudative Vitreoretinopathy](#) (16, 17). Moreover, FZD4 may be involved in transduction and intercellular transmission of polarity information during tissue morphogenesis and tissue differentiation, as its related pathways are [ERK Signaling](#) and [Wnt Signaling](#) pathway (18). Finally, combined with the results of the exome sequencing, we assumed that mutation in the FZD4 might be associated with the congenital cavus foot deformity patients in the Chinese family.

In the present study, three congenital cavus foot deformity patients from one Chinese family (Figure 1) was diagnosed by X-ray and physical examination. Eight people (three patients and five healthy families) was recruited and genetic sequencing was performed in the family. A rare mutation in FZD4 was found in the three patients and none in the healthy families. After that, mutations in FZD4 gene were further examined in twenty-three congenital CFD patients. Our study suggested that the heterozygous mutations in FZD4 (FZD4:NM_012193:exon2 c.1589G>A; p.G530E and exon1:c. 205C>T:p.H69Y) might associated with congenital cavus foot deformity and demonstrated the genotype-phenotype relationship between mutations in the FZD4 gene and characteristics of congenital cavus foot deformity.

Material And Methods

Subject

The total of 3 congenital cavus foot deformity patients and 5 healthy controls in one Chinese Han descent family and 23 unrelated congenital cavus foot deformity patients were recruited to the present study for genetic testing was collected between Aug 2019 and Nov 2020 from the Nanjing Drum Tower Hospital in China. The proband in the family presented with a typical cavus foot deformity phenotype characterized by severe plantarflexion both of the forefoot and the midfoot on the hindfoot. The Hibbs angle and the Coleman bloc test (19), which is a useful method could be employed in the measuring and determining the degree of the cavus deformity. Peripheral whole blood samples were collected for DNA extract and genetic analyses. The chromosomal karyotypes are normal. Bone tissue discarded during surgery in three people hospitalized for traumatic fracture was also collected as the control bone tissue.

Whole-Exome Sequencing and Bioinformatics Analysis

Genomic DNA was extracted from the peripheral blood of the proband and her family members using a Qiagen DNA kit (Qiagen GmbH, Hilden, Germany). Whole exome sequencing (WES) of the proband (2)

and her families (Ⅱ-2, Ⅱ-1, Ⅲ-2 and Ⅲ-1) (Figure 1, Suppl. Fig. 1) was conducted. Briefly, genomic DNA was divided into smaller fragments of 200-250 bp using an ultra-sonic instrument (Covaris LE220; Covaris, Inc., Woburn, MA, USA). Subsequently, purification with Ampure Beads (Beckman Coulter, Inc., Brea, CA, USA) was performed to add poly A/joint reaction to the end of the purified DNA fragments. The gene-trapping chip (Roche NimbleGen, Madison, WI, USA) was used to hybridize with the purified DNA fragments. Captured DNA was sequenced with an Illumina HiSeq2500 Analyzer (Illumina, Inc., San Diego, CA, USA) and read using Illumina Pipeline software (version 1.3.4; Illumina, Inc.). BWA v0.59 (20) was used to align sequence reads to the human genome reference (build 37) and removed duplicated reads from subsequent analyses. Sequence variants were identified via comparisons with the NCBI reference sequence NM 005529.5 and information from 1000 Genomes Project, gnomAD, Esp6500, SIFT and MutationTaster was annotated (21-25).

Previous pedigree analyses indicted that, congenital cavus foot deformity has been assumed to follow an autosomal dominant inheritance. Therefore, we mainly focused on heterozygous rare variants in identified through WES in three patients (patient Ⅱ-2, patient Ⅱ-1 and patient Ⅲ-2, Figure 2A.). Considering the fact that cavus foot deformity leads to mobility impaired and even more serious disabled, the genetic variants with allele frequencies ≥ 0.01 in the human population genome datasets (e.g., the gnomAD Browser and 1000 Genomes Project) were filtered out. Candidate variants were validated by Sanger Sequencing.

Western blot assay and Histological Analysis

Total proteins were extracted from the bone of the feet that was surgically discarded of Patient Ⅱ-2.

RIPA buffer and the bone tissue grinding fluid were subjected to 10% SDS-PAGE and transferred onto PVDF membranes. The membranes were blocked for 2h at room temperature (RT) using 4% nonfat milk-TBST solution and incubated with primary antibodies, antiFZD4 (Abcam, Cambridge, USA), and anti-GAPDH (Bioworld Technology, Minnesota, USA) at 4°C overnight. After successive washes, the membranes were incubated by the goat anti-rabbit HRP-labeled secondary antibodies (Fude Biological Technology Co., Hangzhou, China) for 2h RT. Signals were revealed by Tanon-5200 system (Biotanon, Shanghai, China).

Bone tissue fragments were fixed in 4% paraformaldehyde for 48hs, and placed in a 5% EDTA decalcification solution after washes by water. Then, the tissues embedded in paraffin and serial longitudinal sections (5 μ m thickness) were cut for each sample after dehydrated in an ethanol gradient and cleared with dimethylbenzene. For IHC, hematoxylin was used to counterstain and photographed under a Leica light microscope. Micro-CT (Scanco Medical, Bassersdorf, Switzerland) analyses were performed to evaluate the bone mineral density (BMD) of the heel bone fragments of proband and healthy controls, and a voltage of 70kVp and a current of 114 μ A of the micro-CT scanner was set to collect images at a resolution of 15.6 μ m per pixel. Serial cross-sectional images of the bone fragments were gathered to perform three-dimensional histomorphometric analysis. Bone volume fraction (BV/TV),

trabecular number (Tb.N), trabecular thickness (Tb.Th), trabecular separation (Tb.SP) and bone mineral density (BMD) were obtained by SCANCO Medical software.

Results

Identification of Heterozygous Mutation of FZD4 in Congenital Cavus Foot Deformity Patients

The proband's mother (Figure 1. ♂-2, Patient) was the second child born to healthy parents; there was no cavus foot deformity or other skeletal deformities among other five members. Upon physical examination of the proband. The 20X coverage for the RefSeq coding region was 98.23% (Suppl. Fig. 1). A novel missense mutation (exon2:c.1589G>A;p.G530E) in exon 2 of FZD4 (NM_012193) were detected in every cavus foot deformity patient but not in the healthy families (Figure 2A). One guanine ribonucleotide was altered to an adenine ribonucleotide in codon 1589, which caused a change in the reading frame from glycine (Gly) to glutamic acid (Glu). Sanger sequencing was also performed for this family, which confirmed the heterozygous mutation in the proband and her brother, was inherited by Patient ♂-2, not in any of the healthy families (Fig. 2A). There was no evidence of Cavus Foot Deformity or of other skeletal diseases in the parents of Patient ♂-2. Furthermore, mutations in FZD4 gene were further examined in twenty-three congenital CFD patients, FZD4 mutation (FZD4:NM_012193:exon1:c.205C>T;p.H69Y) in one congenital CFD patient was found (Fig. 2B). In addition, the loci FZD4:p.G530 and p.H69 (NP_036325.2) were highly conserved among a diverse range of species (Figure 2C). The mutations may result in the loss-of-function of FZD4. Both of the two mutations were located in the Topological Domain in FZD4, encoding a transmembrane region (26). In the 1,000 Genomes database (www.internationalgenome.org), Exome Sequencing Project 6500 database (evs.gs.washington.edu/EVS/), and gnomAD genome database (gnomad.broadinstitute.org), the allele frequencies of the c.1589G>A mutation were 0.000998403, 0, and 0.0000956, and c.205C>T mutation were 0.0002, 0.0002, and 0.0005, respectively (Table 1). The SIFT and MutationTaster predicted the mutations as likely to cause damage to the FZD4 protein function (Table 1). To the best of our knowledge, gene FZD4 have not been reported in cavus foot deformity previously. Thus, the present study identified FZD4 mutations as a new gene associated with the congenital cavus foot deformity.

Clinical Characteristic of Congenital Cavus Foot Deformity Patients

The feet in the proband is talipes cavus deformity, which is the most significant complaint, contributing to abnormal elevation of longitudinal arch. The deformity accompanies compound abnormality of the foot, which including adduction of the forefoot, plantar flexion of the midfoot and varus of the hindfoot. Along with the plantar flexion of the forefoot and midfoot is osseous uplift on the dorsal part of the midfoot. There is a subsequent thickening of the skin, with callous formation, under metatarsal heads, result from increasement of bearing under the forefoot. The myodynamia testing of the anterior tibial muscle tendon received a grade of 2/3, which result in weakness of foot dorsiflexion. Consequently, excessive dorsiflexion of toes appears in dorsal expansion position of ankle joint. However, myodynamia of the posterior tibial muscle, peroneus longus and brevis, flexor digitorum longus and flexor digitorum longus is

achieved grade 5. Radiologically, from the lateral radiograph, the M'earny angle of right foot and left foot are 49° and 50° (greater than 10°) respectively, Hibbs angle of right foot and left foot are 35° and 37° (less than 150°) respectively, and calcaneal pitch angle (PTA) of right foot and left foot both are 19mm (greater than 30°).

According to the previous study, bilateral cavus involvement usually suggests an underlying nerve or muscle disease, and 80% of Charcot-Marie-Tooth (CMT) patients having pes cavovarus and 65% of patients with cavovarus having CMT (27). However, the familial cavus patients with this deformity do not have a neuromuscular problem or progressive weakness and muscle imbalance. And according to the clinical and radiographic results, there is no other abnormalities of the spinal cord, such as occult dysraphism, tethered cord, polio, myelodysplasia, and so on. Moreover, peripheral nerves diseases (motor and sensory neuropathies), for example, CMT disease, Dejerine-Sottas disease, or Refsum disease in patients in this study was not found.

Morphological, histological, and IHC analyses of the bone tissue in the cavus foot deformity patient and healthy controls

FZD4 is deficient in the patient's bone tissue. According to Western blot analysis and IHC analyses, the proband's heel bone tissue (CFD) showed a significant deficiency expression of the FZD4 compared to the healthy controls (C1,C2) (Figure 3A). Additional bands below FZD4 was resulted from an important paralog of this gene is [FZD10](#), which is just above 55kb. Micro-CT and X-ray analyses of the bone fragments revealed no significant difference among cavus foot deformity patient and healthy controls in parameters BMD, BV/TV, Tb.N, Tb.SP and Tb.Th (Suppl. Fig. 2). Probably because of the sample size is too small. The result of IHC assessment of bone fragments was in accordance with Western blot analysis, FZD4 shows lighter in the proband's heel bone tissue (CFD) compared to the healthy controls (C1,C2) (Figure 3B).

Discussion

The foot has important impact absorption and ground reaction force transmission functions in bipedal standing position and during gait. Congenital Cavus Foot Deformity is a complex foot deformity rarely seen in clinical practice. There is even no statistics on the incidence of high arches, only morbidity of clubfoot with a worldwide incidence of approximately 1 in 1000 (7). Management of the cavus foot is challenging. As the foot becomes more rigid, surgical options become more limited. Once the foot has lost its flexibility, a midfoot or triple arthrodesis tend to be the mainstays of treatment (28). Early recognition of the deformity and appropriate treatment is important.

Fzd4 is an important factor in bone mineralization. Mice lacking Fzd4 in mature osteoblasts had reduced cortical tissue mineral density and exhibited an impairment in the femoral trabecular bone acquisition, even if there is a normal cortical bone structure, which was due to defection in the mineralization process (15). FZD4 is also a genehancer to heel bone mineral density in GWAS Catalog

(<https://www.ebi.ac.uk/gwas/search?query=FZD4>). However, there's no significant difference in BMD

between the bone fragments of patients and healthy controls calculated by Micro-CT, It may be because the sample size is too small.

Several heterozygote variants in FZD4 have been reported to cause the diseases in the exon 2 region (29, 30) and mutation FZD4:exon2 c.1589G>A;p.G530E (NM_012193) is highly conserved among diverse species, which form a considerable proportion of the reported variants (Fig 1B). Furthermore, the gene encoding Wnt receptor FZD4 can direct activating co-expression of FZD4 and LRP5 receptors, and positively regulate Wnt/bcatenin signaling pathway (13) (Suppl. Fig. 3). The activation of beta-catenin signaling pathway might be signaled by Wnt5a, which further induces osteogenesis. Osteogenesis in the mesenchymal stem cells (MSCs) could be mediated by both canonical and non-canonical Wnt signaling pathways (31-33). In addition FZD4 not only activated components of the Wnt/Ca(2+) signaling pathway, but also CAMK2 and PKC (30). Finally, by combining the specific clinical information, genetic evidence and gene functions, the present study assumed that mutations in gene FZD4 might associated with the congenital cavus foot deformity in the proband family.

There is one interesting founding that worth mention, according to Salvo J (34), the SNV in FZD4:exon2 c.1589G>A; p.G530E (NM_012193) is responsible for Familial Exudative Vitreoretinopathy in one patient. However, there is no eye problems in our congenital cavus foot deformity patients, and their vision is so good that they even don't need to wear glasses. This may consist with the results of clinical test, the associations between NM_012193.4 (FZD4):c.1589G>A (p.G530E) and Familial exudative vitreoretinopathy (Clinical significance: Likely benign) (<https://www.ncbi.nlm.nih.gov/clinvar/RCV000393599.1/#clinical-assertions>) provided by [Illumina Clinical Services Laboratory](#) (Last evaluated: Jun 14, 2016).

Declarations

Ethics approval and consent to participate

This research involving human participants, human material, and human data, were performed in accordance with the Declaration of Helsinki and have been approved by the Ethics Committee of Nanjing Drum Tower Hospital (Jiangsu, China) (The certificate no. 2020-111-02). Written informed consent to participate was obtained from all subjects or their parents/guardians.

Consent for publication

All presentations in this study have consent for publication from all subjects or their parents/guardians.

Availability of data and materials

All data generated or analysed during this study are included in this published article and its supplementary information files.

Competing interests

No, I declare that the authors have no competing interests as defined by BMC, or other interests that might be perceived to influence the results and/or discussion reported in this paper.

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Authors' contributions

Dr. Yan, Dr. Mao, Dr. Xu, Mr. Yang and Prof. Jiang wrote the main manuscript text and Mr. Zheng, Mr. Yu, Dr. Xu prepared figures 1-3 and Table 1. All authors reviewed the manuscript.

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Tables

Due to technical limitations, table 1 is only available as a download in the Supplemental Files section.

Figures

Figure. 1

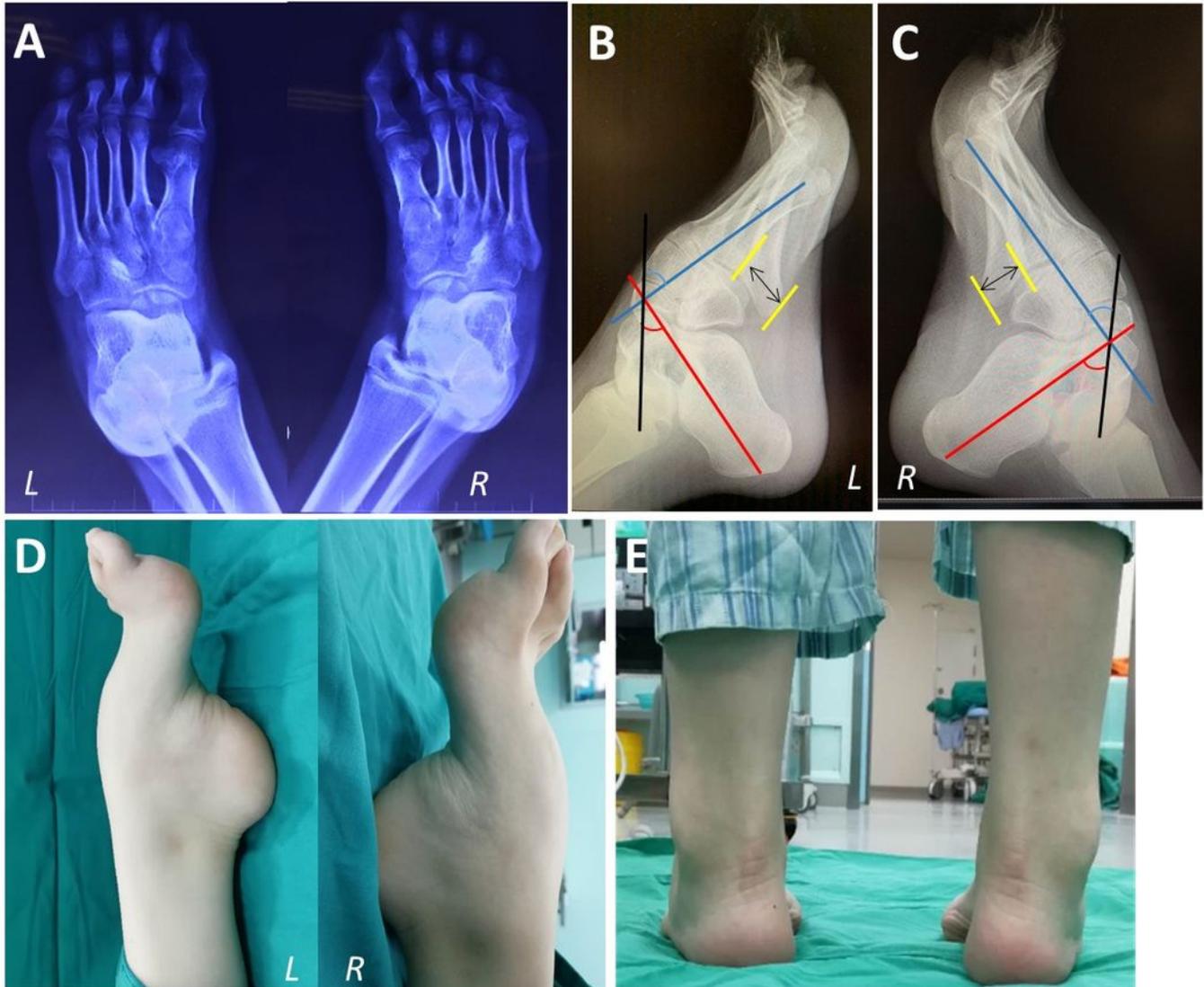


Figure 1

Clinical features of the proband. (A) From the anteroposterior radiograph, calcaneus-talus angle is less than 20° . (B) Left foot. View from lateral. M'earry angle(blue) is 50° , Hibbs angle(red) is 37° , the arch height(yellow), is 19mm; (C) Right foot. View from lateral. M'earry angle(blue) is 49° , Hibbs angle(red) is 35° , the arch height(yellow) is 19mm. (D) and (E) The feet in the proband is talipes cavus deformity, which is the most significant complaint, contributing to abnormal elevation of longitudinal arch. The

suggested that this mutation in each patient were presented and none of this mutation was present in the healthy control. (B). FZD4 mutation c.205C>T: [p.(H69Y)] in one congenital CFD patient. (C).Amino acid alignments in different species around the missense mutation. p.G530 and p.H69 are highly evolutionarily conserved.

Figure. 3

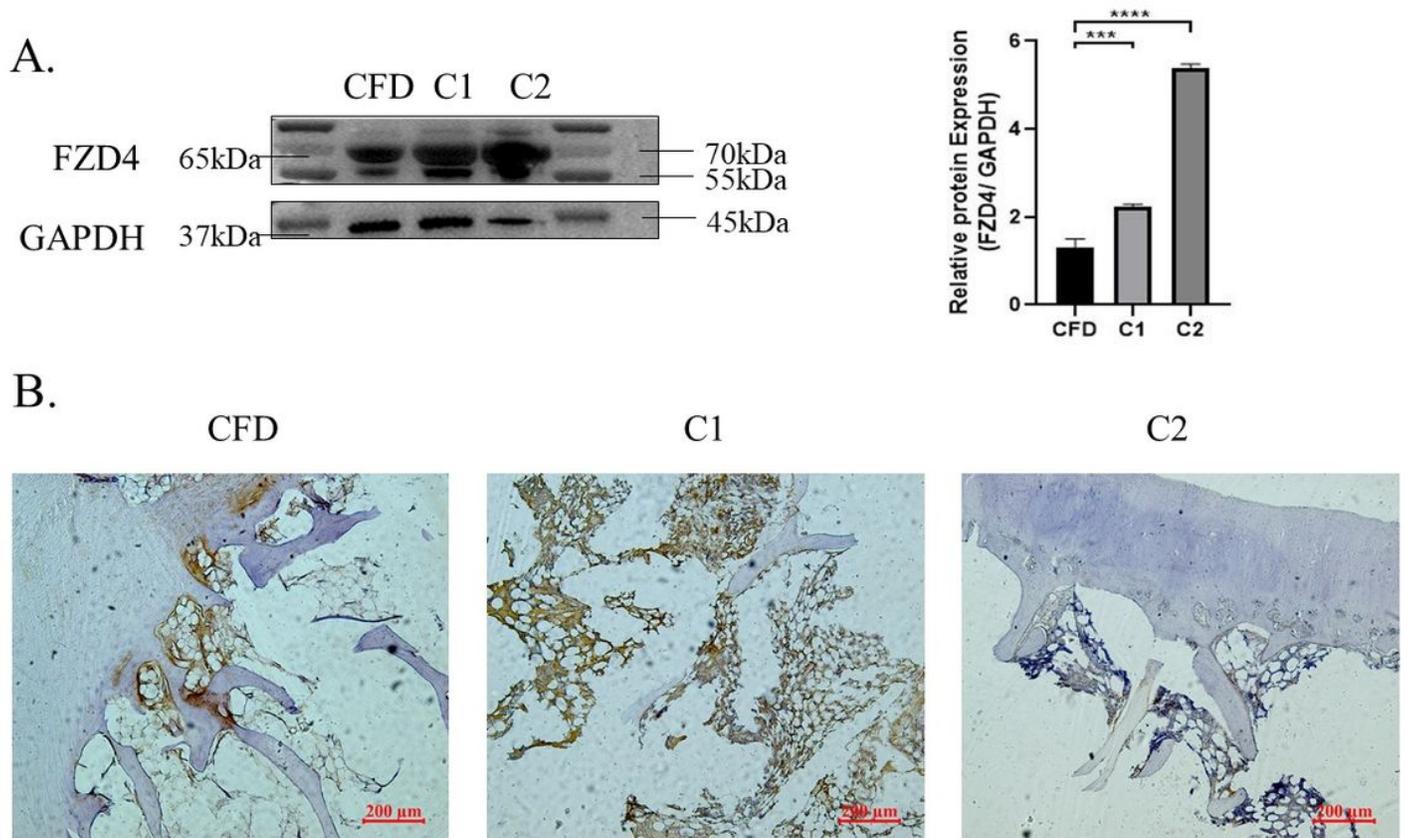


Figure 3

Frizzled Class Receptor 4 (FZD4) expressed in the discard foot tissue during surgery of cavus foot deformity patient (CFD) and healthy controls (C1, C2). (A) Representative Western blot of 3 (n = 3) independent experiments for FZD4 and GAPDH expression. GAPDH was used as internal control. Relative Protein level was determined by density analysis (CFD vs. C1, $p=0.0003$; CFD vs. C2, $p<0.0001$); (B) IHC assessment of bone fragments. Representative IHC images of FZD4. Scale bars, 200 μm .

Supplementary Files

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