Diagnosis with Multiple Epiphyseal Dysplasia Using Whole-exome Sequencing in a Chinese Family

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Introduction

Multiple epiphyseal dysplasia (MED; EDM1, OMIM 132400; EDM2, OMIM 600204; EDM3, OMIM 600969; EDM4, OMIM 226900; EDM5, OMIM 607078; EDM6, OMIM 614135) is an autosomal dominant inherited disease of the skeletal system, characterized by mild short stature and early-onset degenerative joint disease, caused by heterogeneous genotypes involving more than six genes (COMP, COL9A1, COL9A2, COL9A3, MATN3, DTDST). However, in approximately 10–20% of all samples analyzed, a mutation cannot be identified in any of the six genes mentioned above, suggesting that the presence of other unidentified causative genes is also involved in the pathogenesis of MED.\(^1\)

MED can still be difficult to diagnose although it is a relatively common skeletal dysplasia. Avascular necrosis of the femoral head (ANFH, OMIM 608805), Legg-Calve-Perthes disease (LCPD, OMIM 150600), and Beukses familial hip dysplasia (BFHD, OMIM 142669) produce symptoms in hip joint that closely resemble MED. ANFH is a debilitating disease that often leads to disability in adults, genetic studies have indicated that it can be inherited through autosomal dominance (AD).\(^2\) The phenotype is characterized by early onset of ANFH, groin pain, and generalized osteoporosis. The mutation COL2A1 has been associated with ANFH.\(^2\) This mutation has also been shown to cause LCPD, which has ANFH features. LCPD presents with juvenile osteonecrosis of the femoral head, painful limp, delayed bone age, and short stature. BFHD is an autosomal dominant skeletal disorder that has some features of ANFH such as narrowing of the joint space, a shallow acetabulum, and irregular proximal epiphyseal lines between the femur and marginal osteophytes. Mutation in the UFSP2 gene has been identified to be associated with BFHD.\(^3\)

The author found a family, in which multiple members had been diagnosed as ANFH. Eleven affected individuals were found among four generations of this family. Pedigree analysis showed autosomal dominant transmission. Molecular genetic testing was performed on the individuals of this family to provide information for clinical genetic counseling.

Methods

Participants

A 30-year-old woman (IV
_6_ ) from the southern region of Henan Province of China came to our institution for genetic consulting when she was pregnant because she was diagnosed with ANFH at 26 years of age and six other members of her family had ANFH. Upon examination, she was found to have short stature (150 cm high), a waddling...
gait, and hip pain after long walks. Detailed counseling of the patient revealed that 11 members of the family have the same symptoms. The family’s pedigree is shown in Figure 1a.

Informed consent was obtained from all individuals of this family after explaining the nature and possible consequences of the study, and 5 ml peripheral venous blood (EDTA-K2 anticoagulant) was collected from 12 members, including eight patients (II5, III1, III2, IV2, IV6, IV7, IV8, V1) and four unaffected individuals (III3, III6, V2, V5). Twenty milliliter amniotic fluid was collected from proband IV6.

This study adhered to the tenets of the Declaration of Helsinki. Ethical approval was obtained from the Ethics Committee of the People’s Hospital of Henan.

Genetic analysis
Peripheral blood lymphocytes DNA and amniotic fluid DNA were extracted using standard procedures. The whole exon and exon-intron boundaries of the COL2A1 of proband IV6 were amplified and sequenced first. The sequencings were compared with a normal human sequence from the University of California, Santa Cruz (UCSC), 2013 human genome assembly, and no mutation was found. Following that, eight short tandem repeat markers around COL2A1 were used for familial linkage analysis. Co-segregation with phenotype was not found, so the possibility of a mutation in COL2A1 was ruled out.

Whole-exome sequencing (WES) was performed on genome DNA of the proband and individual III3 (exome sequencing and bioinformatic analysis of sequencing raw data completed in Joy Orient Translational Medicine Research Center Co., Ltd.). Variations were annotated using UCSC hg19 refGene. Nonpathogenic variants were removed if they fit the following criteria: (1) variants with an allele frequency more than 1%; (2) variants located in introns and no effect on splicing; (3) synonymous variants without effect on splicing; or (4) variants located in the 5’ or 3’ untranslated region. Finally, 25 common missense mutations in 25 genes were found in proband and individual III1. We reviewed the function and associated OMIM phenotype of those 25 genes. One variant was identified as a candidate pathogenic locus.

Sanger sequencing was used to validate candidate variants. One pair of primers (F: 5’-CCATGAGTTGGGACTCTGT-3’, R: 5’-GGTCAATTCCTGCGGAGTGT-3’) was designed by Oligo Primer Analysis software version 7 (Molecular Biology Insight, Inc., Cascade, CO, USA) to amplify the COMP exon 11 region containing the candidate variants. Polymerase chain reaction (PCR) products were analyzed in 1.5% agarose gels, from which the bands with the amplified product were excised and purified using QIA quick PCR purification kit (Qiagen, Germantown, MD, USA). Sequencing was performed with an ABI BigDye Terminator Cycle Sequencing kit, version 3.1, using an ABI 3100 Genetic Analyzer (Applied Bio Systems, Foster City, CA, USA). Following that, three short tandem repeat markers (D19S-TATG-F: 5’-ACAGAGTTGGGACTCTGT-3’, D19S-TATG-R: 5’-GTAGGCTGAGGTCGAAGAAT-3’, D19S-CTTT-F: 5’-CTCTCTCAAGGAACCTTTC-3’, D19S-CTTT-R: 5’-CAGCCTAGCGACAGAAGG-3’, D19S-TTAT-F: 5’-TTAGCCTTCCCATCCAGTG-3’, D19S-TTAT-R: 5’-TCATGCCTCTAATCCCAAGC-3’) around the COMP gene were used for linkage analysis in the family.

Results
Clinical features
The features of walking instability and waddling gait were

![Figure 1: The pedigree of Chinese family and the result of genetic analysis. (a) Pedigree of the family; *DNA sequence analysis was performed on those family members. The nucleotide position 1153 genotype of coding region COMP and the result of linkage analysis were placed under the symbol, shaded symbols represent the affected individuals, black arrow represents proband, and slanting lines represent deceased individuals. (b) Sequencing results of the exon 11 COMP gene. The upper panel shows heterozygous mutation of c.1153G>A, while the lower panel shows only wild-type alleles.](image)
found in all the affected individuals after 3 years of age. The symptoms of pain in the hip and knee joints after long walks and joint stiffness were observed with age. The height of the affected individuals was in the lower range or slightly shorter, and the hands were shorter than normal. Joint laxity, deformation, and restricted range of movement at the hip joints were found. Individuals II₅, III₅, IV₆, IV₇, and IV₈ were diagnosed with ANFH at 83, 54, 50, 31, 19, and 19 years of age, respectively. The affected individuals II₅ and III₅ cannot walk, and they had not pursued any joint replacement due to lack of resources. The affected individuals IV₆ and IV₇ are twin brothers who have severe symptoms including difficulty with walking. Individuals III₆, IV₆, and IV₇, who have milder symptoms than individuals II₅ and III₅, cannot engage in manual labor because of hip and knee joint pain after moderate activity. The 9-year-old affected individual V₈ presented with unsteady walking, and a waddling gait was observed. No other anomalies were found in this family, the intelligence is normal.

Common radiographic features of individuals II₅, III₅, IV₆, and IV₇ were bilateral flattening of the femoral head and a short femoral neck. Individual II₅ showed blurring of the gap in the hip joints. Individual III₅ presented with a lack of homogeneous bone mineral density, disappearance of the hip gap, and osteophyte formation in the acetabulum. Individual IV₆ showed subluxation of the hip, an irregular and shallow acetabular roof, and cystic and low bone mineral density of the right femur. Individual IV₇ presented with a lack of homogeneous bone mineral density, misplacement of the femoral head to an upper lateral position bilaterally, and a shallow acetabular roof. The radiographic features of individual IV₈, (not pictured) resembled individual IV₇. Radiographic features are shown in Figure 2. The radiographic features of individual V₈ showed a crescent-shaped femoral head with mild flattening, irregularity of the acetabular roof bilaterally, and a broad femoral neck. That individual was diagnosed with bilateral acetalabral dysplasia.

**Mutation analysis**

Among the 25 variants, only four variants (c.1153G>A in COMP, c.704A>G in SYNE2, c.545A>C in EVC, and c.137G>A in FLT4) were associated with an OMIM phenotype which can be inherited through AD (COMP: epiphyseal dysplasia, AD, OMIM: 132400, and pseudoachondroplasia, AD, OMIM: 177170, SYNE2: Emery-Dreifuss muscular dystrophy, AD, OMIM: 612999; EVC: Weyers acrodental dysostosis, AD, OMIM: 193530; FLT4: lymphedema, AD, OMIM: 153100). The variant located in the COMP was identified as a candidate pathogenic locus because the phenotype of the mutation COMP was similar to this family and this variant was a heterozygous missense mutation. Focus was then placed on the heterozygous mutation in COMP. The variant was confirmed by bidirectional Sanger sequencing analysis of exon 11 of COMP in this family. The mutation (c.1153G>A, p.D385N) was detected in all the affected individuals (II₅, III₅, III₆, IV₅, IV₆, IV₇, IV₈, V₅, V₆) and one amniotic fluid DNA sample but was not detected in the four unaffected individuals (III₆, III₇, V₅, V₆) [Figure 1b]. The use of short tandem repeat markers around COMP for linkage analysis showed that the affected individuals carry the same alleles [Figure 1a]. The heterozygous mutation of COMP was co-segregated with phenotype in this family, indicating that the diagnosis of this family’s disease should be MED rather than ANFH.

**DISCUSSION**

This work reported on a large family misdiagnosed with ANFH. Twenty-five pathogenic variants were found by WES, and one variant that occurred in COMP was confirmed by Sanger sequencing and linkage analysis. Originally, COL2A1 was considered to be a candidate pathogenic gene because of clinical data. However, the COL2A1 gene did not co-segregate with phenotype in the eight affected individuals. In the case of the pregnant patient seeking genetic counseling for prenatal diagnosis, it was taken into account that the phenotype of ANFH overlaps among many genes. As a result, WES was used to screen for pathogenic mutations.

The COMP associated with EDM1 is the most common form of MED, accounting for at least half of the cases.[1] MED usually presents with pain in the hips and/or knees after exercise, small height, relatively short limbs in comparison with a normal trunk, pain, and progression of joint deformity. This results in early-onset osteoarthritis, particularly in the large weight-bearing joints, overall delay of maturation of the carpal bones in children, and ANFH in adults or children severely affected with the disease. COMP is also associated with pseudoachondroplasia (PSACH), which has more severe features than MED and is characterized by dwarfism, severe osteoarthropathy, scoliosis, a round ilium, and an irregular acetabulum. In this family, all the affected individuals had pain in the hips and knee joints after long walks and smaller hands and slightly shorter statures. It
is worth noting that the affected individual V₁ showed a crescent-shaped and mildly flattened femoral head, which is a feature of a younger MED patient. The useful diagnostic feature of the “glacier crevice” sign of the knee joints caused by accessory cartilage ossification and typically found in younger MED patients could not be used as it is no longer discernible in adult patients, instead, presented with small epiphyses of the knee joints in adult affected individuals. Unfortunately, these data are not saved in this family; therefore, the femoral head necrosis was diagnosed in this family at first. The mutation c.1153G>A (p. D385N) was reported by Mabuchi et al. in a patient with MED. Given the feature of pain in the knee joints after long walks and smaller hands were not associated with ANFH, combined with genetic diagnosis, this family should ultimately be diagnosed with MED.

COMP protein is a member of the thrombospondin family, and it is found in bone growth plates, tendon bundles, ligaments, and smooth muscles, and consists of an N-terminal domain, four epidermal growth factor-like repeats, eight calmodulin-like repeats (CLRs), and a C-terminal globular domain. CLRs are highly conserved in the COMP amino acid sequence and play a role in binding calcium ions. Aspartate residues are abundant within the CLRs and are considered to function as part of an EF-hand. The majority of disease-causing COMP mutations are clustered in the CLRs, and approximately half of the mutations refer to aspartic acid substitution. Therefore, mutations within the CLRs might affect COMP calcium binding, folding, and stability. The large lamellar rough endoplasmic reticulum (rER) cisternae phenotype is observed in PSACH and MED growth plate chondrocytes. Recent studies found that mutant COMP proteins affect not only COMP secretion but also type IX collagen and MATN3 are selectively sequestered resulting in retention of all the three proteins in the rough endoplasm of PSACH and MED chondrocytes, leading to downstream effects of abnormal matrix and chondrocyte death. This may be the pathogenetic basis of joint disease. The genotype-phenotype correlations revealed that CLRs, and CLRs showed a very significant association with MED compared with the other CLRs, and CLRs, and CLRs were significantly associated with a greater frequency of PSACH compared with mutations in other CLRs. In this report, the pathogenic mutation is located within CLRs, and the clinical feature is manifested in the hip joint lesions.

Other studies found that plasma COMP levels are significantly decreased in patients with COMP mutations compared with controls. This indicated that measuring the level of circulating COMP may be an easier method for differential diagnosis of inherited skeletal dysplasia.

With the wide availability of next-generation sequencing platforms, the cost of sequencing is dropping. Next-generation sequencing has dramatically accelerated biological and biomedical research. WES was first successfully used to identify the genetic mutation of a rare disease by Ng et al. The exome represents approximately 1% of the human genome and is a highly enriched subset of the genome in terms of searching for variants with large effect sizes. Sequencing of the exome, rather than the entire human genome, is a reasonably efficient strategy to search for rare monogenic disorders. Our study illustrates that WES can provide a rapid and cheap way to diagnose a rare disease and thus can serve as a powerful diagnostic tool. However, some limitations to WES should be noted and involve variants lying in the noncoding promoter and intronic sequences, micro-RNA genes, high GC sequences, epigenetic changes, insertion or deletion of small fragments (25–100 bp), translocations, and repeat variants.

In summary, the COMP gene mutation is associated with MED in this family. WES can be used in clinical differential diagnosis.

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Conflicts of interest
There are no conflicts of interest.

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