A genetic pedigree analysis to identify gene mutations involved in femoral head necrosis

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Abstract. The present study presents results from a linkage and mutation screening analysis aiming to identify the causative gene of femoral head necrosis, also known as osteonecrosis of femoral head (ONFH), in a Chinese pedigree. We collected clinical data on the osteonecrosis pedigree, and extracted blood and genomic DNA from the family members. Polymerase chain reaction (PCR) and direct sequencing allowed to identify a mutation in the *COL2A1* gene of the proband; the clinical manifestations of the proband meet the criteria for osteonecrosis. The exons of *COL2A1* were amplified by polymerase chain reaction and mutation screening was conducted by direct sequencing in all the family members. The locus was also sequenced in 50 unrelated healthy controls. The c.3665G>A heterozygous mutation was detected in patients of the pedigree, but not in healthy individuals. We conclude that a mutation in the *COL2A1* gene is the causative agent of ONFH in this family. Therefore, this mutation may be associated with osteonecrosis in Chinese populations.

Introduction

Femoral head necrosis or osteonecrosis of femoral head (ONFH), also known as avascular necrosis, is jointly caused by a variety of disorders of the femoral head blood supply, causing subchondral bone degeneration and necrosis, thus leading to femoral head collapse and eventually, hip joint degeneration (1). The disease occurs mainly in young adults aged 35-55. If untreated, 70% of the patients suffer from femoral head collapse and 3-4 years later total hip replacement is required (2). Therefore, early diagnosis and treatment are key elements in ONFH treatment. In recent years, a variety of osteonecrotic disorders have been associated with genetic polymorphisms, and a high correlation between the incidence of ONFH and these polymorphisms was revealed (3,4). Identification of these polymorphisms by genetic screening before the appearance of clinical symptoms would allow early prevention, diagnosis and treatment of ONFH in vulnerable populations.

The *COL2A1* gene encodes collagen type II α1, a major extracellular matrix component. Type II collagen, also known as cartilage collagen, is the major collagen of cartilage cells. The first step in the synthesis of type II collagen involves three pro-α1 (II) chains twisting together to form a triple-stranded, rope-like procollagen molecule; this procollagen chain contains the N- and C-terminal propeptide of the mature amino acid sequence. Then, the peptide is secreted into the extracellular matrix, and cleaved to form the mature type II collagen molecule (5,6). According to previous reports, mutations in the *COL2A1* gene contribute to numerous diseases, including spinal epiphyseal dysplasia (7), type 2 achondroplasia (8) and Stickler syndrome (9). However, whether pedigree mutations in the *COL2A1* gene may cause disease remains to be studied.

We studied one osteonecrosis pedigree from the province of Anhui, China, and focused on microsatellite markers up- and downstream of the *COL2A1* gene for linkage analysis. We designed primers for PCR amplification of the *COL2A1* gene exons and directly sequenced the products to identify disease-causing mutations. This analysis revealed a mutation potentially causing femoral head necrosis in the studied pedigree, which provides a molecular basis for the diagnosis and prenatal counseling in additional members of the family.

Materials and methods

Subjects. An osteonecrosis pedigree from the Wuwei County in Anhui province, China, was used in this study. The pedigree comprised three generations of a total of 18 Han individuals, 9 of which showed brachydactyly symptoms. The pedigree relationships were confirmed. We obtained written informed consent from all subjects, and collected peripheral blood from 16 individuals, 8 of which were healthy. Representative pictures of bilateral hip X-ray analysis, performed in the 8 patients and the 8 healthy subjects, are shown in Fig. 1.

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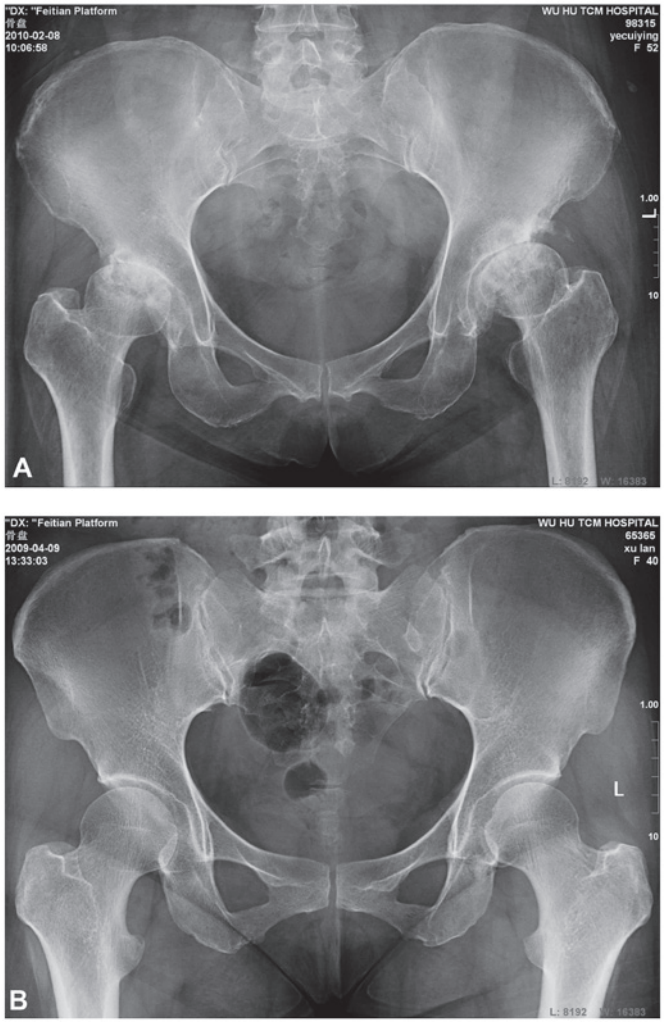

Figure 1. X-ray images of (A) necrotic femoral head and (B) normal femoral head. The gap between the healthy femoral head and the acetabulum is clearly visible, no hardening zone is detected around the femoral head, and its shape is normal. The necrotic femoral head shows signs of collapse, while the direct acetabulum gap is narrow and surrounded by a hardened zone.

Peripheral blood from 50 Han healthy individuals was collected at the Department of Physiology, Medical College of Shantou University, China. These individuals had no symptoms of osteonecrosis and no kinship to the patients.

Nucleic acids isolation. Genomic DNA was extracted from 5 ml of peripheral blood using EDTA as an anticoagulant according to a method previously described with little modification (10). The concentration and purity of the genomic DNA were assessed in a UV spectrophotometer (Tecan, San Diego, CA, USA) at 260/280 nm.

Linkage analysis. Based on the chromosomal location and sequence of the *COL2A1* gene, two polymorphic microsatellite markers were selected, D12S1663 (UniSTS:48691, 12q13.11) and D12S368 (UniSTS:9082, 12q13.13) up- and downstream of the gene for linkage analysis. D12S1663 and D12S368 were amplified using ABI PRISM® Linkage Mapping Set (Applied Biosystems, Foster City, CA, USA). Following PCR, Prism-3100 sequencer (Applied Biosystems) was used to inspect the fragment length for microsatellite genotyping.

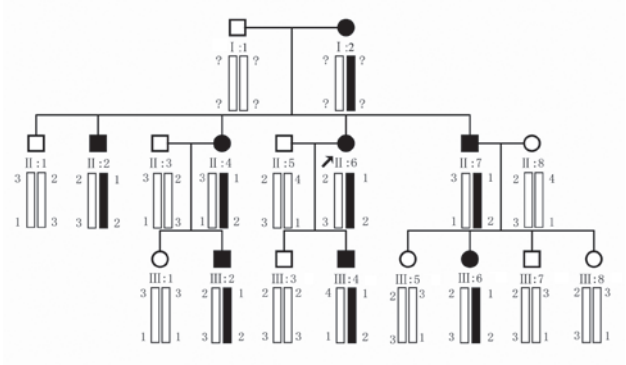

Figure 2. Haplotypes of the three-generation (I-III) Chinese family. Squares denote males and circles females. Black symbols indicate affected individuals, and open symbols unaffected individuals. Arrow, proband; question marks, undetermined; numbers 1-4, different lengths.

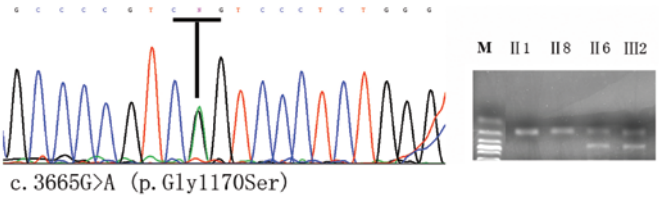

Figure 3. A novel missense mutation in the collagen type II α1 (*COL2A1*) gene causes osteonecrosis of femoral head (ONFH). ONFH was associated with a point mutation at position 3665G>A of the *COL2A1* coding sequence, which causes a G1170S substitution. DNA chromatogram demonstrating the missense mutation c.3665G>A (p.Gly1170Ser) in the proband. M, marker; II1 and II8, unaffected people in the family; II6 and III2, patient.

These two markers were amplified from DNA of the pedigree members, using PCR.

PCR primer synthesis. Primers for the amplification of the two microsatellites were designed based on the sequence of the *COL2A1 gene* (Entrez gene id, 1280; OMIM id, 120140), and were previously described in Liu *et al* (11). The primers were synthesized by the Shanghai Sangon Biological Engineering Technology and Services Co., Ltd. (Shanghai, China).

PCR amplification and sequencing. The PCR reaction was performed in a final volume of 25 µl, comprising 2.5 µl of 10X PCR buffer (Takara Bio, Inc., Shiga, Japan), 2 µl of dNTPs (2.5 mmol/l), 0.1 µl of Taq polymerase (5 U/µl), 0.5 µl of the forward and reverse primer, (50 pmol), 18.4 µl of double distilled water (ddH_2O) and 1 µl of genomic DNA (100-200 ng). The PCR was conducted on a GeneAmp PCR System 9700 (Applied Biosystems) with the following cycling conditions: initial denaturation at 97˚C for 5 min, followed by 35 cycles of denaturation at 95˚C for 45 sec, annealing at 52˚C or 55˚C (for D12S1663 and D12S368, respectively) for 30 sec, extension at 72˚C for 45 sec, and a final extension at 72˚C for 10 min. The PCR products were subjected to denaturing polyacrylamide gel electrophoresis to inspect the fragment length (expected sizes, 223 bp and 115 bp for D12S1663 and D12S368, respectively) for microsatellite genotyping. They were next sequenced by the Shanghai Sangon Biological Engineering Technology and Services Co., Ltd. with a dideoxy-based sequencing protocol.

Results

Symptoms of ONFH in the pedigree. The proband II6 was female, 48 years old and of Han ethnicity. The proband exhibited hip pain upon physical examination, while imaging with X-ray (Fig. 1A) revealed femoral head collapse with a narrow acetabulum gap and a clear hardened zone. However, in the normal control groups (Fig. 1B) the femoral head was normal, the acetabulum gap was clear and there was no hardened zone. The ONFH pedigree comprised three generations (I-III) of a total of 18 people, 9 of which showed brachydactyly symptoms. In all generations, both men and women had the disease, inherited by one of the patient's parents who was also a patient, while healthy offspring was also observed (Fig. 2); this pattern is consistent with an autosomal dominant genetic disease. Physical examination and imaging studies on additional members of the family confirmed that the patients have similar symptoms of ONFH to the proband.

Linkage and COL2A1 gene mutation analysis. Linkage analysis of the *COL2A1* gene in the studied family with the D12S1663 and D12S368STR microsatellites is shown in Fig. 2, and suggested that *COL2A1* may cause ONFH in this family. Sequencing of the PCR-amplified *COL2A1* exon regions in the proband and 8 additional family members revealed that a c.3665G>A heterozygous mutation (Fig. 3) is present in the first exon in seven patients, while this mutation was not detected in 6 normal subjects of the pedigree, indicating that this mutation may associate with the disease. An additional 50 Han individuals not belonging to the pedigree were screened by PCR and sequencing analysis, and the mutation was not identified in any of them. The c.3665G>A mutation causes a glycine-to-serine substitution on codon 1170, which is part of the sequence encoding a GXY repeat in the collagen II protein (Fig. 3).

Discussion

In this study, we identified, by linkage analysis, a mutation in the first exon of the *COL2A1* gene that is potentially associated with necrosis of the femoral head in Anhui pedigrees. Specifically, we found that the genetic basis of the disease in the members of the studied family may be a c.3665G>A mutation (p.G1170S). This mutation, present on the codon 1170 of the human *COL2A1* gene, causes a glycine-to-serine amino acid change in the collagen type II GXY repeat region. The mutation co-segregated with the disease in the pedigree, and was not detected in 50 healthy Han subjects that did not belong to the pedigree, which further supports that the mutation may be pathogenic.

COL2A1 gene mutations can cause a range of serious changes in the encoded product, the collagen type II α1 protein. Chan *et al* found that another *COL2A1* mutation can lead to spinal epiphyseal dysplasia (7). The study of Godfrey and Hollister (12) confirmed one case of perinatal death in the presence of dwarfism caused by a heterozygous mutation in *COL2A1,* resulting in abnormal assembly and folding of type II collagen. Francomano *et al* (13) found, using linkage analysis, that *COL2A1* and the Stickler syndrome are associated. Subsequently in 1990, Ahmad *et al* (9) identified a *COL2A1* mutation in a family with the Stickler syndrome. Knowlton *et al* (14) reported genetic linkage of *COL2A1* to osteoarthritis associated with achondroplasia in a pedigree, followed by Ala-Kokko *et al* (15). Wilkin *et al* (16) identified a *COL2A1* gene mutation in patients with Hackney Manchester hypoplasia; the mutation caused type II collagen defects, rendering the protein shorter. Otospondylomegaepiphyseal dysplasia (OSMED) is a skeletal disorder, associated with severe sensorineural hearing loss, enlarged epiphysis and the early onset of osteoarthritis. Miyamoto *et al* (17) identified a *COL2A1* mutation at a splice-acceptor site within intron 10 in an OSMED patient. Liu *et al* identified three families in which there was autosomal dominant inheritance of the avascular necrosis of the femoral head (ANFH), and mapped the chromosomal position of the gene to 12q13. Haplotype analysis and sequencing of ANFH patients of two pedigrees allowed the authors to identify a G-to-A mutation in exon 50 of *COL2A1*. The mutation was reported to lie on the codon 1170 and to cause a glycine-to-serine replacement in the collagen II GXY repeat region. A G-to-A mutation in exon 33 (codon 717) of the *COL2A1* gene was further identified in a third pedigree, also resulting in a glycine-to-serine replacement. These mutations may lead to an alteration in the structure and function of type II collagen (11). Su *et al* (18) performed mutation screening in a Chinese Han pedigree with osteoarthritis, avascular necrosis, and Legg-Calvé-Perthes disease, and also found a G-to-A mutation in exon 50 of the *COL2A1* gene. In 40-year-old patients with femoral head necrosis, Kannu *et al* (19) identified a novel heterozygous mutation in the *COL2A1* sequence encoding the C-propeptide. Previous studies have shown that mutations in this position generally cause serious limb deformities and even death (20), but the identified mutation in this patient did not cause severe limb deformities.

In conclusion, the present study reports the identification of a *COL2A1* mutation in a Chinese Han pedigree. Although the functions of the COL2A1 protein are understood to a certain degree, the exact mechanisms by which mutations in the gene cause osteonecrosis remain largely unknown. Additional studies on bigger samples and including distinct ethnic backgrounds are required to investigate in depth the pathogenesis of ONFH.

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