

ORIGINAL ARTICLE

Type II Collagen Gene Variants and Inherited Osteonecrosis of the Femoral Head

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ABSTRACT

BACKGROUND

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Avascular necrosis of the femoral head (ANFH) causes disability that often requires surgical intervention. Most cases of ANFH are sporadic, but we identified three families in which there was autosomal dominant inheritance of the disease and mapped the chromosomal position of the gene to 12q13.

METHODS

We carried out haplotype analysis in the families, selected candidate genes from the critical interval for ANFH on 12q13, and sequenced the promoter and exonic regions of the type II collagen gene (*COL2A1*) from persons with inherited and sporadic forms of ANFH.

RESULTS

We identified a G→A transition in exon 50 of *COL2A1* in affected members of a four-generation family with ANFH. This transition predicts the replacement of glycine with serine at codon 1170 in a GXY repeat of type II collagen. Another pedigree was shown to harbor the same transition, but the mutant allele occurred on a different haplotype background. In a third family, a G→A transition in exon 33 of the gene, causing a glycine-to-serine change at codon 717, was detected. No mutation was found in the *COL2A1* coding region in sporadic cases of ANFH.

CONCLUSIONS

All the patients with familial ANFH whom we studied carried *COL2A1* mutations. In families with ANFH, haplotype and sequence analysis of the *COL2A1* gene can be used to identify carriers of the mutant allele before the onset of clinical symptoms, allowing the initiation of measures that may delay progression of the disease.

IT HAS BEEN ESTIMATED THAT 300,000 TO 600,000 people in the United States have avascular necrosis of the femoral head (ANFH).¹ Approximately 15,000 new cases of this common and disabling disorder, also called ischemic necrosis of the femoral head or osteonecrosis of the femoral head, are reported annually.² The age at the onset of this disease is earlier than that for osteoarthritis; the diagnosis is typically made when patients are between the ages of 30 and 60 years. The clinical manifestations, such as pain on exertion, a limping gait, and a discrepancy in leg length, cause considerable disability. Moreover, nearly 10 percent of the 500,000 total-hip arthroplasties performed each year in the United States involve patients with ANFH.³ As a result, this disease creates a substantial socioeconomic cost as well as a burden for patients and their families.

Idiopathic osteonecrosis of the femoral head is defined as a disease that causes ischemic osteonecrosis of the femoral head without trauma or sepsis, according to the proposal put forth by the Association Research Circulation Osseous in 1993.⁴ In this definition, so-called steroid-induced osteonecrosis and alcohol-associated osteonecrosis are also included in this category. Although the exact pathogenesis of osteonecrosis remains unknown, it appears to involve vascular compromise, cell death, or deficient bone repair.⁵

We recently identified three families with idiopathic ANFH in which there was autosomal dominant inheritance of the disease and reported that the familial ANFH gene mapped to a 15-cM region between *D12S1663* and *D12S1632* on chromosome 12q13.⁶ To identify the genes associated with idiopathic osteonecrosis, we scrutinized the region by resequencing the coding and promoter regions of candidate genes.

METHODS

RECRUITMENT

The pedigrees of the three families with ANFH are shown in Figure 1. The disease was diagnosed in affected persons by clinical and radiologic criteria. The chief symptom reported by most of these patients was pain in the groin. Physical examination revealed that the patients had no other skeletal anomalies. Routine plain-film radiography of the pelvis and hip joints was used for ANFH staging, according to Ficat's classification.⁷ The genetic study was approved by the institutional research

board of the National Health Research Institutes, Taipei, Taiwan. Written informed consent was obtained from all persons who contributed DNA and clinical information to the study. Both affected and unaffected family members were Han Chinese living in Taiwan; their ancestral origin was determined by the investigators on the basis of geography and language.

GENOTYPING AND HAPLOTYPE ANALYSIS

Genotyping was performed with leukocyte DNA for 39 microsatellite repeat markers from chromosome 12 (Table 1 of the Supplementary Appendix, available with the full text of this article at www.nejm.org). Reactions were performed in 96-well microtiter plates; each well contained 10 ng of DNA template, 6 μ l of a polymerase-chain reaction (PCR) amplification mix (True Allele PCR Premix, Applied Biosystems), and 0.06 pmol of each primer in a final volume of 10 μ l. The PCR products were analyzed on an ABI Prism 3700 DNA sequencer (Applied Biosystems). GeneScan version 3.6 and Genotyper version 3.5 (Applied Biosystems) for Windows NT were used for allele identification and size assignment. Construction of the haplotypes was performed by the GeneHunter program.⁸

DNA SEQUENCE ANALYSIS

All exons of the type 2 collagen gene (*COL2A1*) from core members of the families (Subjects III-7, III-8, IV-5, IV-6, and IV-7 in Family A; Subjects III-7, III-8, and IV-10 in Family B; and Subjects II-7, II-8, III-12, and III-13 in Family C) were analyzed. In addition, genomic PCR and sequencing of exons 33 and 50 were performed on DNA from all available family members, 65 persons with sporadic ANFH, and 110 controls. Leukocyte DNA was used to amplify the genomic fragments of the *COL2A1* promoter as well as exons and exon-intron junctions in 39 PCR reactions. Oligonucleotide primers were designed with the use of Primer3 software⁹ (Table 2 of the Supplementary Appendix). PCR was initiated at 95°C for 10 minutes; this step was followed by 45 cycles of 95°C for 30 seconds, an annealing temperature for 30 seconds, and 72°C for 45 seconds. The final step took place at 72°C for 3 minutes. PCR products were treated with exonuclease I to remove excess primers. The DNA sequencing reaction was performed with the Dye Terminator kit (Applied Biosystems) with the same primers as were used in the PCR amplification, and the reaction products were separated with ABI 3700 or ABI 3730 DNA sequencers.

The results were analyzed with the Phred/Phrap/Consed programs¹⁰⁻¹² (www.phrap.org) and PolyPhred software (version 10).¹³

Allele-specific primer extension was used to confirm patients' heterozygous state. Amplification of exon 50 wild-type sequence was carried out with primer A (5'-AGTCAGGACACTTACAGCAG-3') plus primer B (5'-GTCCTCCTGGCCCCGTCG-3'), and amplification of exon 50 mutant sequence with primer A plus primer C (5'-GTCCTCCTGGCCCGTCA-3'). Amplification of exon 33 wild-type sequence was carried out with primer A (5'-TCAGTGGGACTCCCAGGC-3') plus primer B (5'-GTGCCAGGGCCTCCAGG-3'), and amplification of exon 50 mutant sequence, with primer A plus primer C (5'-GTGCCAGGGCCTCCAGA-3').

RESULTS

CLINICAL CHARACTERISTICS

Three families with ANFH were recruited for the current study (Fig. 1). Two of them (Families A and B) have been described previously.⁶ Family C was subsequently added, but only the core family members were available for the current investigation. In conjunction, 65 consecutive patients with sporadic idiopathic ANFH were enlisted during the period from July 2003 to May 2004.

Of the three families with ANFH, there were 32 living members (11 males and 21 females) in whom the disorder had been diagnosed at the time the study was initiated. Twelve members of Family A (4 male and 8 female), 11 members of Family B (6 male and 5 female), and 3 members of Family C (1 male and 2 female) were available for clinical and genetic analyses, and their profiles were compared with those of the 65 patients with sporadic ANFH (55 male and 10 female) (Table 1). Although the sex ratios in the two groups were quite different, we believe that this discrepancy was probably due to ascertainment bias, since we recruited more male patients than female patients from a veterans' hospital. The inherited form of ANFH was associated with a younger age at onset, bilateral hip involvement, and no apparent predisposing conditions. Approximately 85 percent of the patients with sporadic disease had one or more complicating factors, including systemic lupus erythematosus, alcohol consumption, or use of steroid medication. Consumption of alcohol was the single most important nongenetic risk factor associated with the sporadic ANFH.

Figure 1 (facing page). Pedigrees of the Three Families with ANFH.

Panel A shows the pedigree and results of haplotype analysis of Family A. Short tandem-repeat markers spanning the candidate region for ANFH were used to construct the haplotype. The short tandem-repeat markers used to identify recombination breakpoints and to define the critical region are shown in red, as are the identifying numbers of the subjects involved in that analysis. Asterisks indicate subjects who carried a mutant *COL2A1* allele that was clinically silent at the initial assessment. Panel B shows the pedigree and results of haplotype analysis of Family B. Asterisks indicate subjects who may have carried the mutant allele. Because of space constraints, two affected female subjects whose DNA was not available for haplotype analysis have been omitted from the diagram. Panel C shows the pedigree of Family C. The red box encloses the four core members available for DNA sequence analysis.

The clinicopathologic features of a 24-year-old patient are shown in Figure 2. The patient, Subject IV-5 in Family A, presented with groin pain when she was 16 years old. A hip-joint radiograph obtained when she was 21 years old revealed a Ficat's stage II lesion (Fig. 2A), and a magnetic resonance image (MRI) obtained at the same time revealed apparent necrosis of the bone of the femoral head (Fig. 2B). Core-decompression surgery was performed at the age of 21 years. Histopathological examination of the specimen revealed necrosis of bone and marrow tissue — findings consistent with the diagnosis of ANFH (Fig. 2C).

FINE MAPPING OF THE CANDIDATE REGION

To investigate the molecular mechanisms underlying idiopathic osteonecrosis, we took a positional candidate approach to isolate the mutated gene in the families with autosomal dominant ANFH. We first conducted haplotype analysis in two families in which linkage to chromosome 12 had been found and further defined the candidate region for ANFH. In Family A, recombination events were evident in Subjects III-20 and IV-2, and the breakpoints helped to narrow the ANFH-critical region to an 8.2-cM interval between *D12S1301* and *D12S339* (Fig. 1A). We inferred that this genomic segment might harbor the gene for the disease in this family. The affected members of Family B shared, within the family, a haplotype in the ANFH-critical region. However, this disease-associated haplotype, defined by markers between *D12S1663* and *D12S2196*, differed from that of the affected members of Family A. We did

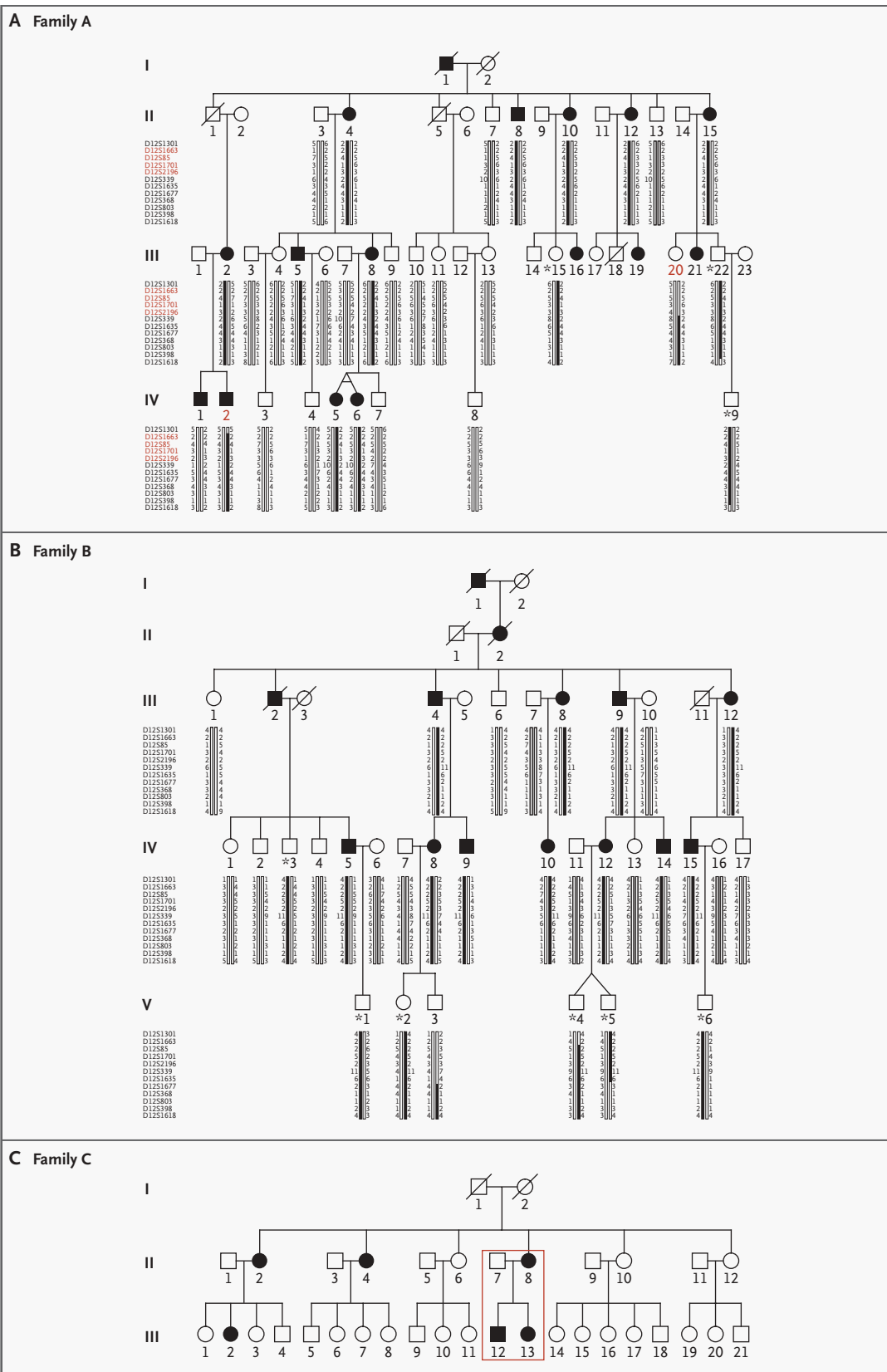


Table 1. Characteristics of the Patients with Familial or Sporadic ANFH.

Characteristic	Family A (N=12)	Family B (N=11)	Family C (N=3)	Sporadic (N=65)
Sex*				
Male	5	8	1	55
Female	11	8	5	10
Age (yr)				
<1–30	4	1	1	8
31–60	4	8	2	50
61–80	4	2	0	7
Associated conditions				
None (idiopathic)	12	11	3	10
Systemic lupus erythematosus	0	0	0	10
Steroid use	0	0	0	6
Alcohol use	0	0	0	31
Steroid and alcohol use	0	0	0	8
Involved site				
Both hips	12	11	3	36
One hip	0	0	0	29

* Affected persons who were not available for clinical evaluation are included in the data on sex.

not carry out haplotype analysis of the third family with ANFH (Family C).

COL2A1 GENE MUTATIONS IN FAMILIAL ANFH

Using Genome Browser (National Center for Biotechnology Information, build 33, April 2003), we identified 21 known genes and 25 predicted genes in the 5.13-Mb critical region between *D12S1301* and *D12S339*. Genes with functions related to bone and cartilage physiology were considered candidates for autosomal dominant ANFH. Among them, *COL2A1* was mapped to 12q13.11–q13.2; it encodes type II collagen, a major structural protein in the extracellular matrix of cartilage. Since type II collagen provides mechanical strength in the cartilage, a mutation in the *COL2A1* gene might be the cause of the hip-joint disorder in the affected families.

We applied a resequencing strategy to investigate this possibility and to uncover any genetic alterations in *COL2A1* that may be associated with ANFH. A 3665G→A transition (GenBank accession number NM_001844) that caused a change from glycine to serine at amino acid position 1170 (GenBank accession number NP_001835) was identified in all 12 available patients from Family A and all 11

available patients from Family B (Fig. 3A). In all the available patients from Family C, a 2306G→A transition (GenBank accession number NM_001844) was identified (Fig. 3B); this transition resulted in a change from glycine to serine in the GXY repeat at amino acid position 717 (GenBank accession number NP_001835) of the predicted protein. The two mutations were not found in either the 110 controls or the 65 persons with sporadic ANFH whom we analyzed. To confirm the heterozygous state of the sequenced mutations, we conducted additional DNA-based diagnostic tests by means of allele-specific primer extension (Fig. 3C) and heteroduplex analysis with the use of single-strand conformational polymorphism and denaturing high-performance liquid chromatography techniques (Fig. 1 and 2 of the Supplementary Appendix). All the assay results are consistent with the conclusion that the patients have a mutant allele as well as a wild-type allele.

DISCUSSION

In this study, we succeeded in using a positional candidate approach to identify the *COL2A1* gene from the chromosome 12q13 region as the gene that is mutated in the inherited form of ANFH. Three points lend support to the idea that the identified mutations in the *COL2A1* gene might be the cause of the disease in the three families we examined. First, the *COL2A1* gene transitions segregated with the disease in these families. Second, the variant sequence did not occur in 220 chromosomes of 110 controls examined. Third, the amino acid change falls at a critical position in the GXY triple-helix repeat of the encoded collagen molecule. Thus, we conclude that the structural alteration in the GXY repeat domain of type II collagen contributes to the pathogenesis of autosomal dominant ANFH.

The clinical features of the three multiplex families are similar to those of the sporadic cases of ANFH seen more commonly in orthopedic practice. Except for the hip joints, the affected persons were otherwise normal, with average height and normal spine development (Fig. 3 of the Supplementary Appendix). Moreover, they had no anomalies of the ocular or auditory system. Notably, the *COL2A1* gene transitions found in the three families with ANFH result in a glycine-to-serine amino acid change in the GXY repeat of the collagen (Fig. 3D). We speculate that, unlike other type II collagenopathies (which are caused by deletion of the gene, trunca-

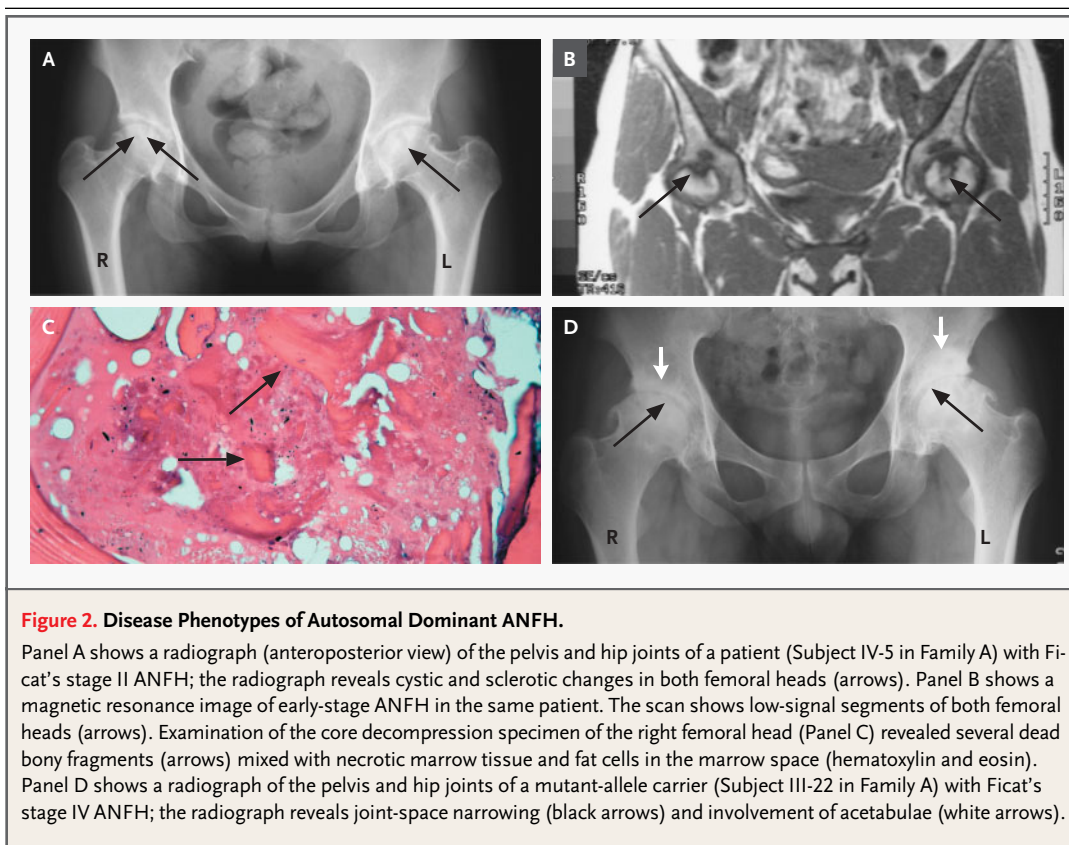


Figure 2. Disease Phenotypes of Autosomal Dominant ANFH.

Panel A shows a radiograph (anteroposterior view) of the pelvis and hip joints of a patient (Subject IV-5 in Family A) with Ficat's stage II ANFH; the radiograph reveals cystic and sclerotic changes in both femoral heads (arrows). Panel B shows a magnetic resonance image of early-stage ANFH in the same patient. The scan shows low-signal segments of both femoral heads (arrows). Examination of the core decompression specimen of the right femoral head (Panel C) revealed several dead bony fragments (arrows) mixed with necrotic marrow tissue and fat cells in the marrow space (hematoxylin and eosin). Panel D shows a radiograph of the pelvis and hip joints of a mutant-allele carrier (Subject III-22 in Family A) with Ficat's stage IV ANFH; the radiograph reveals joint-space narrowing (black arrows) and involvement of acetabulae (white arrows).

tion of the protein, or substitution with a bulky amino acid),¹⁴ the *COL2A1* transitions in familial ANFH might have a milder effect on the structure and function of the protein.

A glycine-to-serine substitution has been reported in several inherited skeletal disorders involving type II collagen, including achondrogenesis or hypochondrogenesis,¹⁵⁻²⁰ spondyloepiphyseal dysplasia,²¹⁻²⁵ and osteoarthritis²⁶ (Table 3 of the Supplementary Appendix). It is intriguing that the transitions reported here are distinct from other *COL2A1* mutations and that they result in glycine-to-serine alterations at different positions in the triple-helix domain. Moreover, it is apparent that glycine-to-serine amino acid substitutions in this domain can give rise to diseases with different clinical presentations and varying degrees of severity. It remains to be determined how glycine-to-serine alterations at different positions could affect the post-translational processing of type II collagen and, consequently, contribute to variable pathophysiological and clinical manifestations. Perhaps, depending on its location in the GXY repeat domain, a specific type of glycine-to-serine substitution

might change the fibril structure and interfere with the molecular interactions with other extracellular matrix constituents, such as proteoglycans. In addition, the expression level of the mutant allele relative to the wild-type allele in specific tissues could also have an effect on triple-helix composition and, consequently, clinical severity.

DNA-sequence analysis of the *COL2A1* gene in members of Family A allowed us to identify persons at risk for ANFH. Among 19 persons who had no symptoms at the initial medical examination, 16 had G/G homozygosity at nucleotide position 3665, whereas 3 (Subjects III-15, III-22, and IV-9) had a G→A transition. These three subjects also carried the same haplotype in the critical interval between *D12S1301* and *D12S339* as the patients with ANFH. Groin pain subsequently developed in two of the three subjects (III-15 and III-22, both adults), and a recent radiograph of the hip in Subject III-22 revealed late-stage ANFH (Ficat's stage IV) (Fig. 2D). We conclude that although there is individual variability in the age at onset and in tolerance of the symptoms of the disease, the results of DNA testing coincide with the clinical diagnosis. Moreover,

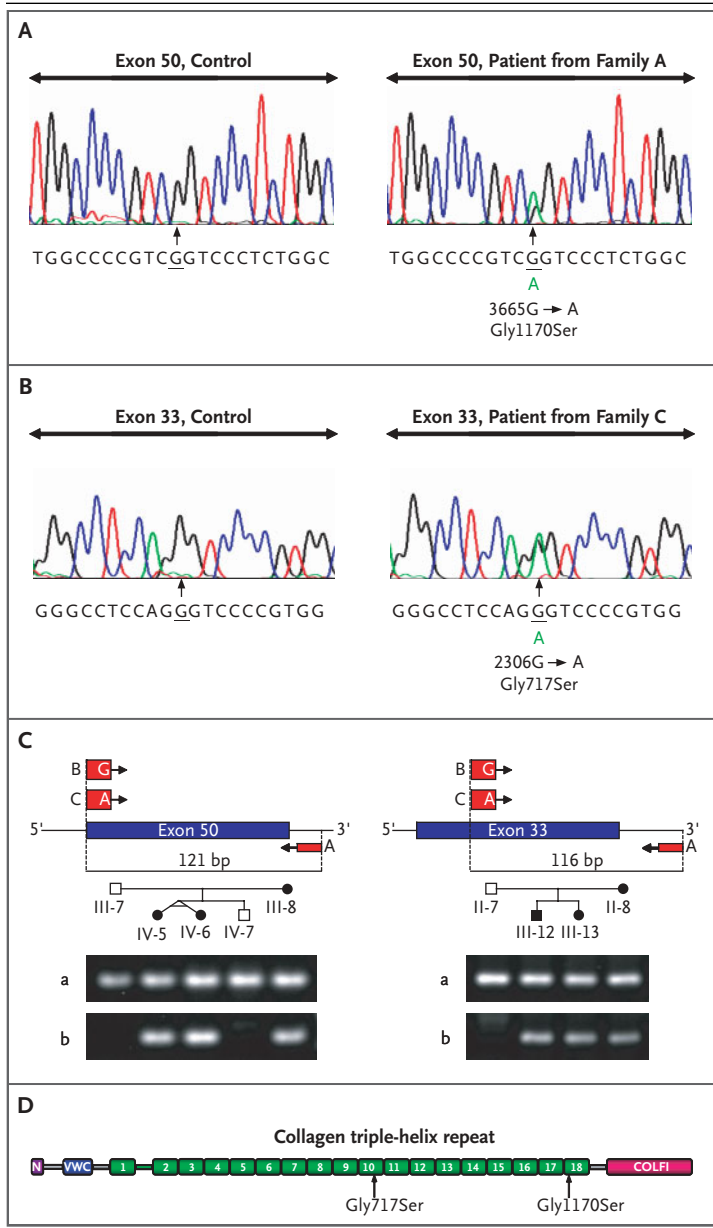


Figure 3. COL2A1 Mutations in Families with ANFH.

Direct sequencing of fragments amplified by the polymerase chain reaction revealed a 3665G→A substitution in the mutant allele from a patient in Family A (Panel A) and a 2306G→A substitution in the mutant allele from a patient in Family C (Panel B). Panel C shows the results of allele-specific primer extension of the wild-type and G→A mutant sequences. DNA samples annealed with primer pair AB (subpanel a) amplified the wild-type allele and those with primer pair AC (subpanel b) amplified the mutant allele. Affected family members (Subjects III-8, IV-5, and IV-6 of Family A and Subjects II-8, III-12, and III-13 of Family C) were heterozygous for the mutant COL2A1 sequence. In both reactions, primer A was anti-sense-strand wild-type sequence, primer B sense-strand wild-type sequence, and primer C sense-strand mutant sequence. Panel D is a schematic representation of type II collagen, showing the positions of the glycine-to-serine mutation in the triple-helix repeat. VWC denotes von Willebrand factor C domain, and COLFI fibrillar collagen C-terminal domain. The diagram is based on annotated messenger RNA of the COL2A1 gene from the Information Engineering Branch of the National Center for Biotechnology Information (www.ncbi.nlm.nih.gov/IEB/Research/Acembly).

of presymptomatic carriers of ANFH (Subjects III-15, III-22, and IV-9 in Family A and Subjects IV-3, V-1, V-2, V-4, V-5, and V-6 in Family B) provides an opportunity to study the natural history of the disease. Given the genetic basis of the disease, it is now possible to apply radiographic and metabolic indexes, such as measurement of the concentration of collagen fragment in the blood or urine,²⁷ to monitor the progression of the disease.

The discovery of COL2A1 as the “causative” disease gene underlying inherited ANFH is unexpected. As its name implies, ANFH is attributed to a compromised vascular supply, but type II collagen is a marker for chondrocytes. Furthermore, mutation of the COL2A1 gene is known to be the cause of congenital disorders of the skeletal system. The results of the current study, however, suggest that defective cartilage function or abnormal bone homeostasis due to COL2A1 mutations could also play a role in the pathogenesis of osteonecrosis of the femoral head.

Several strategies have been tested for the preservation of the femoral head in patients with osteonecrosis, including surgical procedures²⁸ and medication with osteoinductive or angiogenic agents²⁹ to enhance bone formation and repair.³⁰ The goal of these approaches is either to reduce the intraosseous

COL2A1 haplotype and sequence analysis can potentially allow presymptomatic diagnostic testing to be offered to members of families with ANFH.

The third subject (IV-9), currently six years old, does not have the disease, and the most recent radiograph showed normal development of the hips and spine (Fig. 3C of the Supplementary Appendix). As we found in our previous genetic study of two ANFH kindreds,⁶ the development of hip-joint problems in carriers of the COL2A1 mutant allele did not occur until adulthood. Thus, the identification

pressure in the femoral head, and thereby restore normal vascular flow, or to enhance regional vascularization. It is known that core decompression for osteonecrosis of the hip has an effect on the natural history and clinical progression of the disease when necrotic involvement of the femoral head is limited.²⁸ Therefore, molecular analysis of the COL2A1 gene can be used in families with ANFH to provide an indication for intervention when there is an early sign of osteonecrosis.

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