Am. J. Hum. Genet. 64:1036–1044, 1999

COL9A3: A Third Locus for Multiple Epiphyseal Dysplasia

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Summary

Multiple epiphyseal dysplasia (MED), an autosomal dominant osteochondrodysplasia, is a clinically and genetically heterogeneous disorder characterized by mild short stature and early-onset osteoarthritis. The phenotypic spectrum includes the mild Ribbing type, the more severe Fairbank type, and some unclassified forms. Linkage studies have identified two loci for MED. One of these, *EDM1,* is on chromosome 19, in a region that contains the cartilage oligomeric matrix protein (COMP) gene. Mutations have been identified in this gene in patients with the Ribbing type, the Fairbank type, and unclassified forms of MED. The second locus, *EDM2,* maps to chromosome 1, in a region spanning *COL9A2.* Recently, a splice-site mutation was found in *COL9A2,* causing skipping of exon 3 in one family with MED. Because of the exclusion of the *EDM1* and *EDM2* loci in some families, the existence of a third locus has been postulated. We report here one family with MED, evaluated clinically and radiologically and tested for linkage with candidate genes, including *COMP, COL9A1, COL9A2,* and *COL9A3.* No linkage was found with *COMP, COL9A1,* or *COL9A2,* but an inheritance pattern consistent with linkage was observed with *COL9A3.* Mutation analysis of *COL9A3* identified an A→T transversion in the acceptor splice site of intron 2 in affected family members. The mutation led to skipping of exon 3 and an in-frame deletion of 12 amino acid residues in the COL3 domain of the α3(IX) chain and thus appeared to be similar to that reported for *COL9A2.* This is the first disease-causing mutation identified in *COL9A3.* Our results also show that *COL9A3,* located on chromosome 20, is a third locus for MED.

Received September 30, 1998; accepted for publication February 4, 1999; electronically published March 17, 1999.

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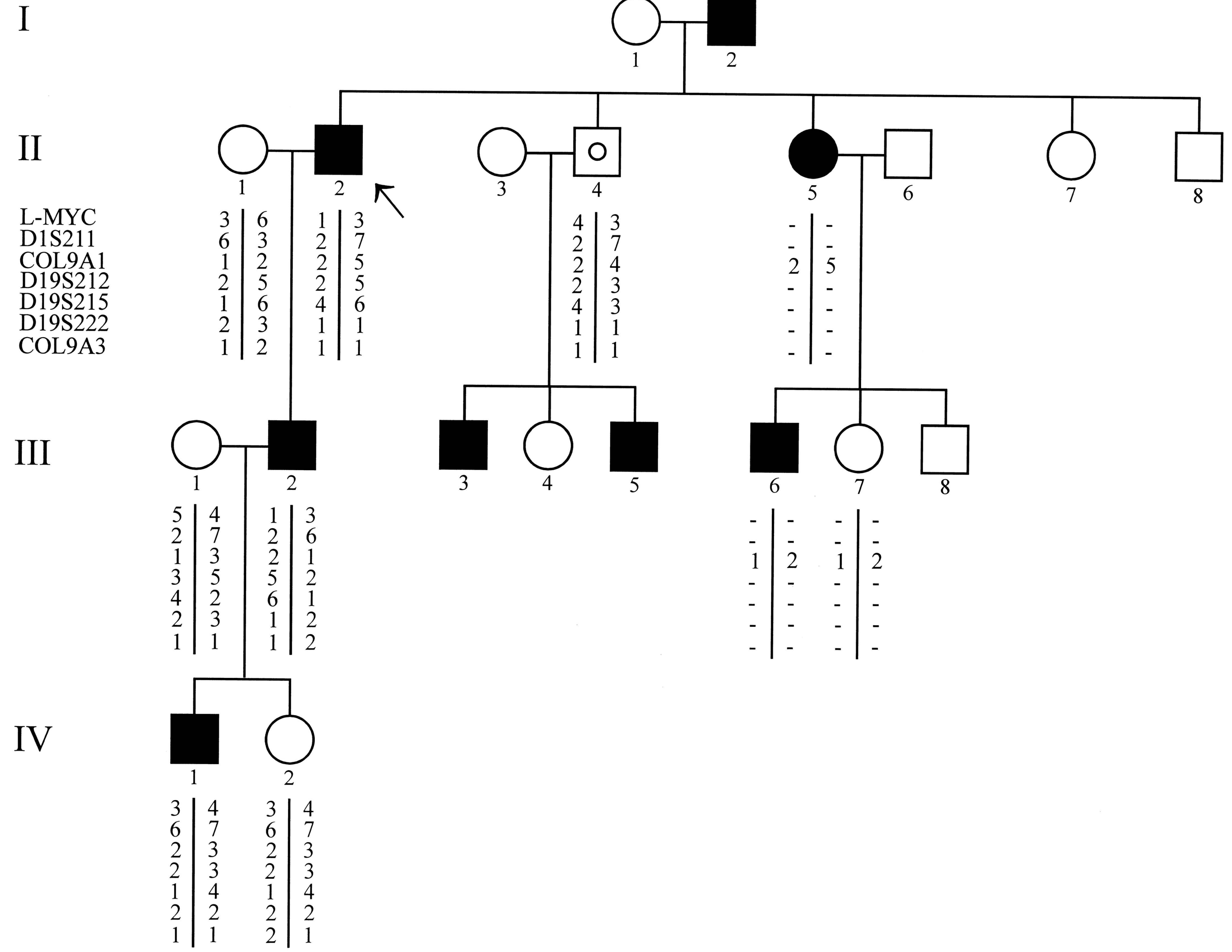
0002-9297/99/6404-0015$02.00

Introduction

Multiple epiphyseal dysplasia (MED; MIM 132400) is an autosomal dominantly inherited osteochondrodysplasia characterized by mild-to-moderate short stature and early-onset osteoarthritis. MED consists of the clinically overlapping Ribbing and Fairbank phenotypes as well as other forms (International Working Group on Constitutional Diseases of Bone 1998). The symptoms include pain and stiffness of multiple joints during childhood and adolescence and development of osteoarthritis in late childhood to adulthood. Affected individuals usually have a slightly shorter stature than their unaffected siblings, and they may have short, stubby fingers. Radiographic features show irregular and flattened epiphyses with delayed ossification. The vertebrae and metaphyses are usually unaffected (Weaver et al. 1993; Rimoin et al. 1994). Some patients have been reported to have only radiological evidence of MED or osteoarthritis in the absence of complaints (Oehlmann et al. 1994; van Mourik et al. 1998).

In addition to its clinical heterogeneity, MED is also a genetically heterogeneous disease. It can be caused by mutations in the cartilage oligomeric matrix protein (COMP; MIM 600310) gene (*EDM1*; MIM 132400), or in the *COL9A2* gene that encodes the α2 chain of collagen IX (*EDM2;* MIM 600204). *COMP* is located on chromosome 19 (Newton et al. 1994) and *COL9A2* is located on chromosome 1 (Perälä et al. 1993). Mutations resulting in Ribbing, Fairbank, and unclassified forms of MED have been identified in *COMP* (Briggs et al. 1995; Cohn et al. 1996; Ballo et al. 1997; Susic et al. 1997; Briggs et al. 1998; Loughlin et al. 1998). Furthermore, EDM1 and pseudoachondroplasia (PSACH) have proved to be allelic disorders because mutations in *COMP* have also been identified in patients with PSACH (Briggs et al. 1995, 1998; Hecht et al. 1995; Maddox et al. 1997; Susic et al. 1997; Loughlin et al. 1998). PSACH is more severe than MED, but the phenotypes of its mild forms overlap with those of MED (Maroteaux et al. 1980; Wynne-Davies et al. 1986; Rimoin et al. 1994; Briggs et al. 1998).

Collagen IX is a heterotrimeric nonfibrillar collagen


I
II
III
IV
L-MYC
D1S211
COL9A1
D19S212
D19S215
D19S222
COL9A3

consisting of three different α chains, α1(IX), α2(IX), and α3(IX), that are encoded by three distinct genes, *COL9A1, COL9A2,* and *COL9A3* (van der Rest and Mayne 1987; Shaw and Olsen 1991). Each polypeptide chain consists of three collagenous domains, COL1–COL3, interrupted by four noncollagenous domains, NC1–NC4. The domains are numbered from the C terminus. Collagen IX is a structural component of hyaline cartilage, intervertebral discs, and the vitreous body of the eye. Its precise function is not well established, although a possible role in the stabilization of the fibril network has been suggested (Bruckner et al. 1988). Results obtained with transgenic mice suggest that collagen IX is needed for the long-term structural integrity of cartilage (Nakata et al. 1993; Fässler et al. 1994; Haimes et al. 1995). Muragaki et al. (1996) and van Mourik et al. (1998) reported that a splice-site mutation in *COL9A2* has been shown to cause MED of an unclassified form in one family. This mutation caused skipping of exon 3 and thus in-frame deletion of 12 amino acid residues in the COL3 domain of the α2(IX) chain.

Linkage with the EDM1 and EDM2 loci has been excluded in one family with MED, which suggests the existence of at least one additional locus (EDM3; MIM 600969; Deere et al. 1995). We searched for additional loci by analyzing a family with an unclassified form of MED for linkage with candidate genes, including *COMP, COL9A1, COL9A2,* and *COL9A3*. Whereas linkage with *COL9A1, COL9A2,* and *COMP* was excluded, no recombination was observed between the phenotype and *COL9A3*. Mutation analysis of *COL9A3* identified a splice-site mutation leading to an in-frame deletion of 12 amino acid residues in the COL3 domain of the α3(IX) chain. This is the first disease-causing mutation to be identified in *COL9A3*. The results also show *COL9A3* to be a third locus for MED.

Subjects and Methods

Subjects

Members of a four-generation family with MED were studied. Clinical and radiologic examinations were performed on six affected family members and four unaffected ones. After informed consent, blood samples were taken from the family members, for DNA analysis. Blood samples were also obtained from 65 healthy volunteers, for DNA analysis.

Linkage Analysis

Intragenic markers were used to perform linkage analysis on *COL9A1* and *COL9A3*. A tetranucleotide repeat in intron 12 of *COL9A1* had been reported elsewhere (Pihlajamaa et al. 1998). Exon 5 of *COL9A3,* which contains a neutral G→A sequence variation (CGG→CAG), was amplified with the PCR primers FI5, 5′-CACCAAGGGAAGGGTCCGTGC, and RI5, 5′-CTACCAGCTCCTTGGCCTTGTGG (Paassilta et al., in press). PCR amplifications were performed in a reaction volume of 30 μl containing 60 ng genomic DNA, 5 pmol each primer, 200 μM dNTP, 1.5 mM MgCl, and 1 U *Taq* polymerase (AmpliTaq Gold, Perkin-Elmer). The DNA samples were amplified for 35 cycles at 94.5°C for 40 s, 60°C for 50 s, and 72°C for 1 min, followed by one extension cycle at 72°C for 10 min. Allelic inheritance of the intragenic markers was analyzed by conformation-sensitive gel electrophoresis (CSGE). Microsatellite markers were used to analyze linkage to chromosome 19 (*D19S212, D19S215,* and *D19S222*) and to chromosome 1 (*D1S211* and *L-MYC*). The sequences of the primers and the conditions for PCR amplification have been described elsewhere (Mäkelä et al. 1992; Weissenbach et al. 1992).

PCR Amplification of COL9A3

The PCR primers were designed to amplify all 32 exons and exon boundaries of *COL9A3* (Paassilta et al., in press). The lengths of the PCR products varied from 209 to 411 bp, and each contained ⩾70 bp of the exon-flanking sequences. Genomic DNA was amplified in a volume of 40 μl with thermal cycling of 45 s at 94°C, 45 s at 60°–63°C, and 1 min at 72°C, for 30 cycles, and 10 min at 72°C for 1 cycle, followed by denaturation at 95°C for 5 min and incubation at 68°C for 30 min, to generate heteroduplexes. The PCR products were analyzed on 1.5% agarose gels.

Heteroduplex Analysis

The CSGE method was used to analyze the PCR products for the presence of heteroduplexes. A CSGE gel was prepared with 10% polyacrylamide, 99:1 ratio of acrylamide (Intermountain Scientific) to 1,4-bis(acryloyl) piperazine (Fluka), 10% (vol/vol) ethylene glycol (Sigma), 15% (vol/vol) formamide (Gibco), 0.1% ammonium persulphate (U.S. Biochemicals), and 0.07% N,N,N′,N′-tetramethylethylenediamine (Sigma) in 0.5 ×

Figure 1 Pedigree of family with MED. Blackened symbols indicate affected individuals. The proband is marked by an arrow. Subject II-4 (shown by circle within open square) did not have any complaints, and the results of a physical examination were normal. The affected status could not be determined by roentgenogram analysis, because radiologic examination was refused. Alleles of the indicated markers are shown below the symbols.

Table 1

Clinical and Radiological Findings

Subject	Sex	Year of Birth	Height (cm)	Area of Clinical Complaint	Results of Clinical Examination	Affected Joints (radiographs)
II-2	M	1932	168	Knees, hips	Limited extension of knees and elbows, replaced left hip, valgus knees	Knees, hips
II-4	M	1942	171	Minor knee pain	No abnormalities	Not done
II-5	F	1945	160	Knees, hips	Limited extension of knees	Knees
III-2	M	1957	169	Severe knee pain	Limited extension of knees and hips, mild valgus knees	Knees, hips, ankles, wrists
III-3	M	1967	180	Knees	Knee crepitation	Knees
III-5	M	1979	187	Knees	Knee crepitation	Knees, hips
III-6	M	1968	Not known	Knees	Not done	Not done
IV-1	M	1986	148	Severe knee pain	Limited extension of elbows, hips and knees, valgus knees	Knees, hips, ankles, hands

TTE (44 mM Tris/14.5 mM Taurine/0.1 mM EDTA, [pH 9.0]) buffer. A standard DNA-sequencing apparatus was used for gel electrophoresis with 0.5 × TTE as the electrode buffer. The gel was preelectrophoresed at 45 W for 15 min. A total of 50–75 ng PCR products were mixed with loading buffer (10 × stock solution of 30% glycerol containing 0.25% both xylene cyanol FF and bromphenol blue), and the mixture was loaded on a CSGE gel. The samples were electrophoresed for 5 h at room temperature at 45 W, after which the gel was stained with ethidium bromide and photographed (Ganguly et al. 1993; Körkkö et al. 1998).

Sequencing of PCR Products

The samples that contained heteroduplexes were treated with exonuclease I and shrimp alkaline phosphatase by incubation at 37°C for 15 min followed by inactivation at 80°C for 15 min. They were then analyzed by automated sequencing (ABI PRISM model 377 Sequencer and dRhod Dye Terminator Cycle Sequencing Ready Reaction Mix, Perkin-Elmer).

RNA Analysis

RNA was extracted from Epstein-Barr virus (EBV)–transformed lymphoblasts obtained from an individual in the control group, EBV-transformed lymphoblasts obtained from a proband, and primary cultured chondrocytes obtained from one affected family member during hip surgery, by the acid guanidium thiocyanate/phenol/chloroform method (Chomczynski and Sacchi 1987). The Superscript Preamplification System (Gibco BRL) with random hexamers was used to synthesize the first-strand cDNA. PCR primers corresponding to exons 1 (E1F, 5′-CGCGCCGCTCCTGCTCCTGCTCCTC) and 9 (E9R, 5′-ACCAGGGAGGCCGGGGAGTCCAGA) of the α3(IX) cDNA (Brewton et al. 1995) were used for the first PCR amplification, whereas the second was performed by use of nested PCR primers from exons 1 (E1Fb, 5′-GGCGGCCGCCGGGGCGCAGAGA) and 6 (E6R, 5′-GGGCGGCCCCGGGGGTCCCAGACTTC). The PCR amplifications took place in a volume of 50 μl with 1 μl of the PCR template, 10 pmol each primer, and 1 U *Taq* polymerase. Thermal cycling conditions included one cycle at 95°C for 2 min and 35 cycles at 95°C for 40 s, 58°C–62°C for 50 s, and 72°C for 1 min, followed by one extension cycle at 72°C for 10 min. The products from the second PCR reaction were analyzed on 4% agarose gels, cloned into the pUC 18 vector (SURE Clone Ligation Kit, Pharmacia), and sequenced.

Results

Clinical and Radiological Findings

A four-generation family with autosomal dominant osteochondrodysplasia MED was studied (fig. 1). All the affected family members experienced knee pain and stiffness during childhood. They all experienced difficulties in walking as a result of these symptoms, and some developed hip arthrosis and required surgical hip replacement after age 50 years. The affected adults had normal hands and no spinal symptoms, and their height was normal (−1 SD to + 1 SD) and did not differ from that of the unaffected family members. The phenotype of the family members varied(table 1).

Radiographs of the male proband II-2 taken at age 59 years showed irregular and slightly narrow epiphyses of the knees (not shown). Radiographs of his hips showed narrowing of the joint spaces and osteophyte formation. The capital femoral epiphyses were smaller than normal, and the acetabuli were slightly hypoplastic (not shown). The proband had left hip replacement at age 63 years. Radiographs of subject III-5 (a nephew of the proband), taken at age 16 years, indicated small irregularities of the lateral condyles of the distal femoral epiphyses and slight narrowing of the internal compartment (not shown). Radiographs of his hips showed mild condensation of the acetabulum (not shown). Ra-

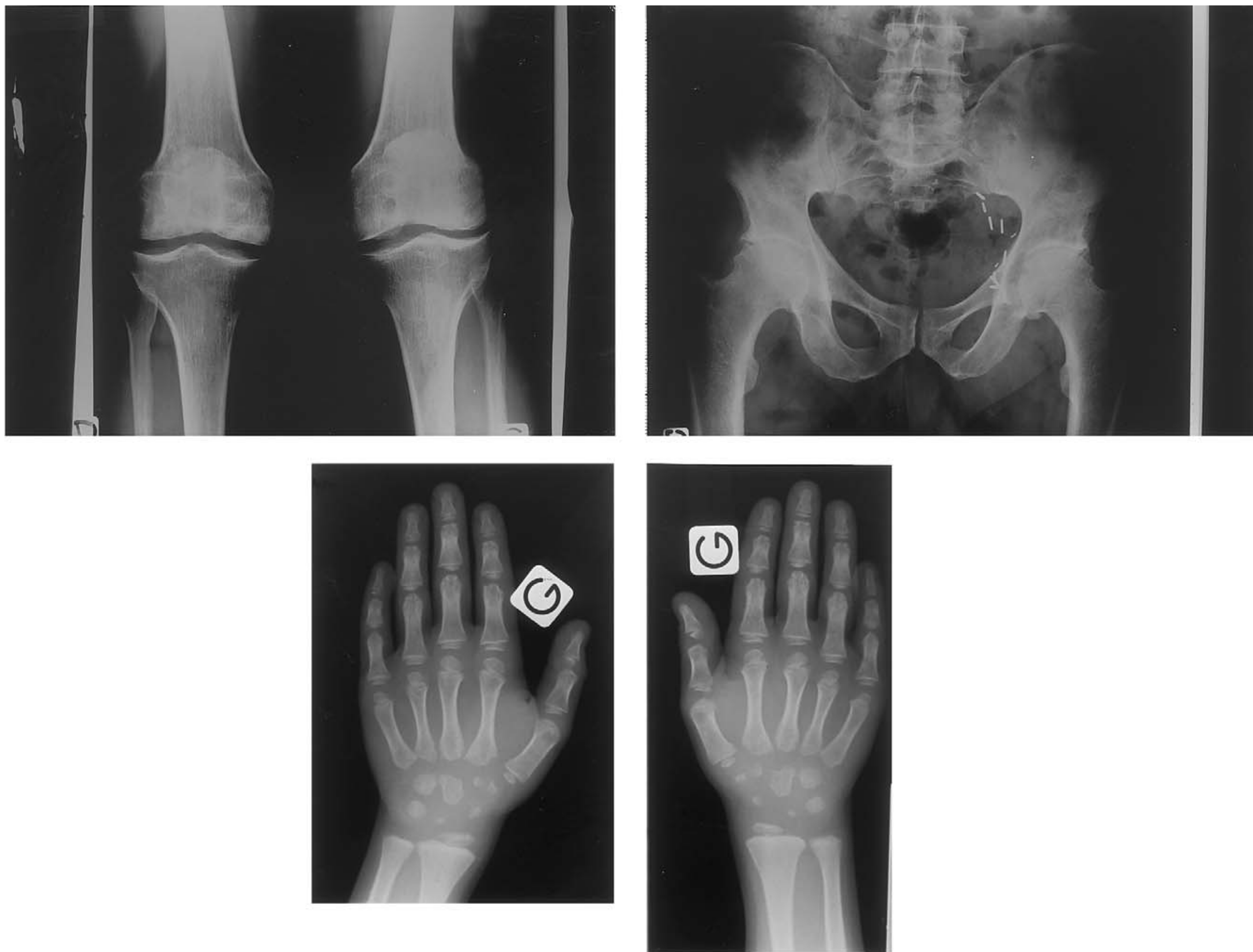


Figure 2 Roentgenograms of the knees (*A*), pelvis (*B*), and hands (*C*) of subject IV-1

diographs of subject III-2 (the proband's son), taken at age 12 years, showed osteochondritis dissecans of the left femoral condyle and irregularities of the lower epiphyses of the knees, the carpal bones, and the epiphyses of the ankle (not shown). The proband's grandson, subject IV-1, showed flattened, irregular, and fragmented distal femoral epiphyses at age 8 years (fig. 2*A*). His hip epiphyses were flat and underdeveloped (fig. 2*B*), the carpal bones were irregular and small, and the metacarpal bones were shortened (fig. 2*C*). Radiographs of the spine showed normal findings (not shown). Although the radiologic findings of the adult affected family members showed mild dysplasia, the diagnosis of MED was established on the basis of the radiographs of the younger individuals. Clinical and radiologic findings for most of the affected family members are shown in table 1. One family member, subject IV-2, showed multiple exostoses at the knee level, without any signs of MED.

Linkage and Mutation Analysis

Linkage with four candidate genes, *COMP, COL9A1, COL9A2,* and *COL9A3,* was tested. Recombination was observed between *COMP, COL9A1,* or *COL9A2* and the MED phenotype in this family, but an inheritance pattern consistent with linkage was found between the phenotype and an intragenic polymorphism in the *COL9A3* gene (fig. 1). Two affected family members (subjects II-2 and III-2) and two unaffected ones (subjects III-1 and IV-2) were analyzed for mutations in *COL9A3*. The PCR products of all the exons and exon boundaries were analyzed with CSGE, and those that contained heteroduplexes in CSGE were sequenced. CSGE analysis of exon 3 showed a unique heteroduplex in both affected family members but not in the unaffected members. Six additional family members were analyzed for the presence of the heteroduplex, which was

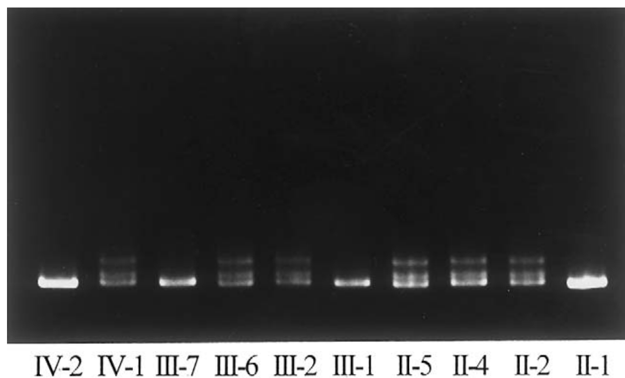


Figure 3 CSGE analysis of exon 3. Heteroduplexes were detected in all the affected family members (seefig. 1).

coinherited by all the affected members (figs.1and 3). Sequencing of exon 3 showed an A→T mutation at the acceptor site (A^{-2}IVS2→T) of intron 2 (fig. 4*A*). The mutation was not present in DNAs from unaffected family members (fig. 4*B*). In addition, the mutation was not found in 130 control alleles. The presence of the mutation in all the affected family members was confirmed by sequencing and by digestion with *Tsp*RI restriction endonuclease (data not shown).

RNA Analysis

To study the consequences of the mutation for mRNA splicing, total RNA was prepared from EBV-immortalized lymphoblasts from the proband (subject II-2) and from an individual in the control group. In addition, RNA was extracted from cultured chondrocytes from the proband's affected sister, subject II-5. RNA samples were reverse transcribed (RT) and PCR products from exons 1–9 were generated. Nested primers were used for the second PCR, to amplify the PCR products from exons 1–6. Two major products of ~280 bp and ~240 bp were obtained from lymphoblast RNA (fig. 5*A*) and chondrocyte RNA (not shown). Control RNA gave one product of ~280 bp (fig. 5*B*). The sequence of the ~280-bp PCR product corresponded to that of the wild-type allele (data not shown). Sequencing of the ~240-bp PCR product indicated a skipping of exon 3 and thus deletion of 36 nucleotides (fig. 6).

Discussion

Linkage studies have suggested at least three loci for MED, namely EDM1, EDM2, and a third unidentified locus (Briggs et al. 1994; Oehlmann et al. 1994; Deere et al. 1995). Here we excluded linkage to the EDM1 and EDM2 loci in a family with MED, but no recombination was observed between the disease and a third locus, *COL9A3* (EDM3), on chromosome 20 (Brewton et al. 1995). Several MED mutations have been described in *COMP,* corresponding to the EDM1 locus (Briggs et al. 1995; Cohn et al. 1996; Ballo et al. 1997; Susic et al. 1997; Briggs et al. 1998; Loughlin et al. 1998), and one in *COL9A2,* corresponding to the EDM2 locus (Muragaki et al. 1996). We now describe a splice-site mutation in *COL9A3,* leading to skipping of exon 3 and an in-frame loss of 12 amino acid residues in the COL3 domain of the α3(IX) chain. The consequence of this mutation for the α3(IX) chain is identical to that reported for the α2(IX) chain (Muragaki et al. 1996).

The sizes of the domains and the amino acid sequences of the α1(IX), α2(IX), and α3(IX) chains are highly conserved (Pihlajamaa et al. 1998), and they form heterotrimeric molecules that consist of three α chains in a 1:1:1 ratio. Because *COL9A2* and *COL9A3* mutations can cause MED, one could expect *COL9A1* to be another locus for MED. The phenotype caused by the *COL9A3* mutation is similar to that caused by the mutation in the *COL9A2* gene, thus emphasizing the po-

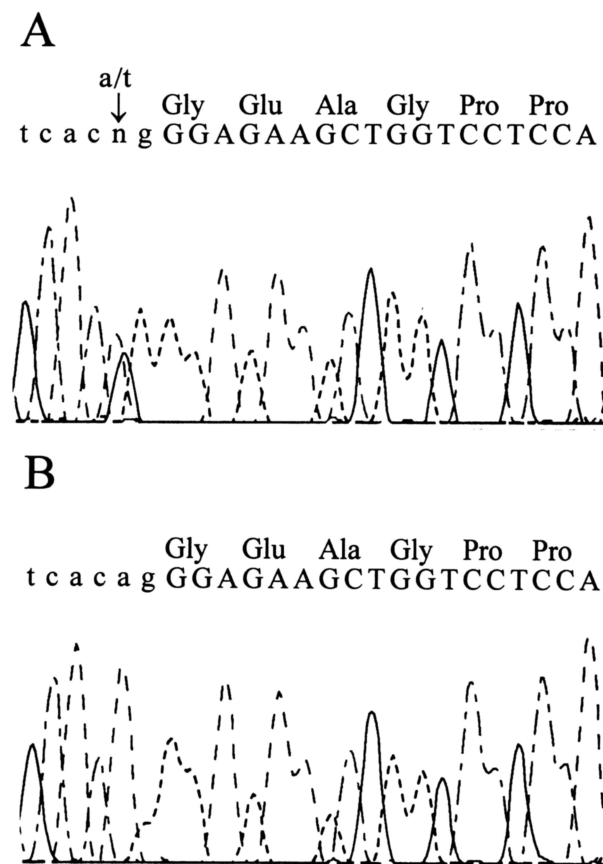


Figure 4 Sequencing of the PCR products from exon 3. *A*, Sequence of the mutated allele. Transversion of A→T (A^{-2}IVS2→T) is indicated by an arrow. *B*, Sequence of the wild-type allele.

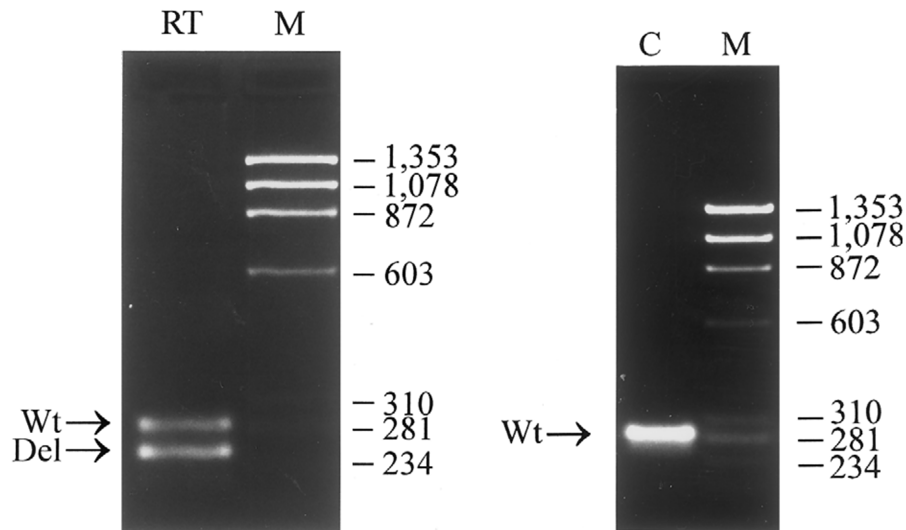


Figure 5 Agarose gel electrophoresis of $\alpha3$(IX) RT-PCR products. RNA was isolated from the EBV-transformed lymphoblasts of the proband (*A*) and a control individual (*B*) and used for cDNA synthesis. The second PCR was performed with exon 1 and six specific nested primers. Two PCR products were obtained, a 280-bp product corresponding to the wild-type allele (Wt) and a 240-bp product corresponding to the allele with a deletion (Del). "RT" denotes RT-PCR products of the proband; "M" denotes molecular weight marker; and "C" denotes RT-PCR product of the control sample.

tential role of the COL3 domain in the pathogenesis of MED. Initial association of collagen α chains occurs through the C-terminal ends of the molecules (Engel and Prockop 1991; Mechling et al. 1996) and is followed by triple helix formation that progresses from the C terminus toward the N terminus. Hence, the effect of the deletion in the COL3 domain is likely to propagate toward the N terminus and may thus alter the structure and function of the most N-terminal portion of the molecule.

The Ribbing type of MED includes flat epiphyses, leading to early-onset osteoarthritis in the hips and short stature. The more severe Fairbank type is characterized by dwarfism, stubby fingers, and small epiphyses of several joints, including the hips (Ribbing 1937; Fairbank 1947; Barrie et al. 1958; Silverman 1996). The wide variability in MED phenotypes nevertheless makes classification difficult, and many families with MED remain unclassified because of the absence of typical Ribbing or Fairbank features. So far, mutations in *COMP* and *COL9A2* have been shown to cause MED phenotype. The phenotype caused by a mutation in *COL9A2* does not exhibit significant involvement of the hip joints and seems to be milder than the phenotypes caused by mutations in *COMP.* (Weaver et al. 1993; Briggs et al. 1994; van Mourik et al. 1998). The MED phenotype described here is also relatively mild and closely related to the phenotype caused by a mutation in *COL9A2,* because the affected individuals were of normal or slightly short stature and showed involvement of the knee joints. How-

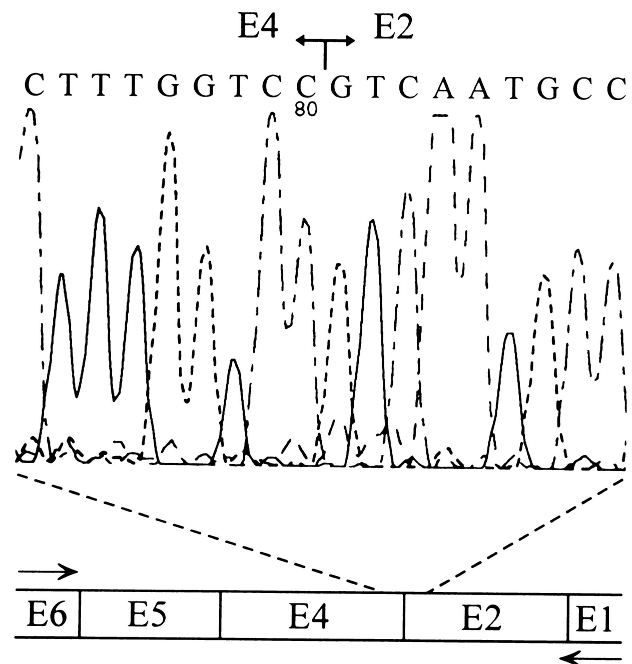


Figure 6 Sequence of the 240-bp RT-PCR product (fig. 5*A*), which was cloned to the pUC 18 vector. Sequencing with reverse primers showed deletion of the sequences for exon 3. The vertical line (*top*) indicates the boundary between exons 2 and 4. The boxes (*bottom*) indicate the cloned cDNA region (drawn to scale). The PCR primers used for the second PCR are indicated with arrows (*bottom*).

ever, some individuals with the *COL9A3* mutation showed significant hip involvement. The *COL9A3* mutation thus results in a phenotype that overlaps with the phenotype caused by the *COL9A2* mutation but differs from it in the presence of hip involvement. Because the *COL9A2* and *COL9A3* mutations affect the respective collagen chains in identical ways, differences in the phenotypes could be a result of additional factors (genetic or environmental) that modulate the phenotypic severity and account for the intrafamilial variability seen in MED (van Mourik et al. 1998). The results suggest that phenotypes caused by collagen IX mutations could be milder than ones caused by *COMP* mutations. Identification of additional mutations, however, is needed to define the full clinical spectrum of MED caused by collagen IX mutations.

Acknowledgments

We thank Dr. Björn R. Olsen for helpful discussions and comments and Ms. Aira Harju for her expert technical assistance.

Electronic-Database Information

Accession numbers and URL for data in this article are as follows:

Online Mendelian Inheritance in Man (OMIM), http://www.ncbi.nlm.nih.gov/Omim (for MED [MIM 132400] COMP [MIM 600310], EDM1 [MIM 132400], EDM2 [MIM 600204], and EDM3 [MIM 600969])

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