A Mutation in *COL9A1* Causes Multiple Epiphyseal Dysplasia: Further Evidence for Locus Heterogeneity

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Multiple epiphyseal dysplasia (MED) is an autosomal dominantly inherited chondrodysplasia. It is clinically highly heterogeneous, partially because of its complex genetic background. Mutations in four genes, *COL9A2, COL9A3, COMP,* and *MATR3,* all coding for cartilage extracellular matrix components (i.e., the α2 and α3 chains of collagen IX, cartilage oligomeric matrix protein, and matrilin-3), have been identified in this disease so far, but no mutations have yet been reported in the third collagen IX gene, *COL9A1,* which codes for the α1(IX) chain. MED with apparently recessive inheritance has been reported in some families. A homozygous R279W mutation was recently found in the diastrophic dysplasia sulfate transporter gene, *DTDST,* in a patient with MED who had a club foot and double-layered patella. The series consisted of 41 probands with MED, 16 of whom were familial and on 4 of whom linkage analyses were performed. Recombination was observed between *COL9A1, COL9A2, COL9A3,* and *COMP* and the MED phenotype in two of the families, and between *COL9A2, COL9A3,* and *COMP* and the phenotype in the other two families. Screening of *COL9A1* for mutations in the two probands from the families in which this gene was not involved in the recombinations failed to identify any disease-causing mutations. The remaining 37 probands were screened for mutations in all three collagen IX genes and in the *COMP* gene. The probands with talipes deformities or multipartite patella were also screened for the R279W mutation in *DTDST*. The analysis resulted in identification of three mutations in *COMP* and one in *COL9A1,* but none in the other two collagen IX genes. Two of the probands with a multipartite patella had the homozygous *DTDST* mutation. The results show that mutations in *COL9A1* can cause MED, but they also suggest that mutations in *COL9A1, COL9A2, COL9A3, COMP,* and *DTDST* are not the major causes of MED and that there exists at least one additional locus.

Introduction

Multiple epiphyseal dysplasia (MED [MIM 132400]) is a disorder of the skeletal system that is manifested as a disturbance in the development of the epiphyses (Fairbank 1947), the major component of which is cartilage. The changes are usually observed in the majority of the growth centers, including those of the spine (Hoefnagel 1967). The joints are usually bilaterally—but not always symmetrically—affected in MED.

The first symptoms of MED occur in childhood, usually at age 2–14 years, and include waddling gait, restriction of joint mobility, and pain and stiffness in the weight-bearing joints. MED is clinically heterogeneous, consisting of the Fairbank, Ribbing, and unclassified types (International Working Group on Constitutional Diseases of Bone 1998). The Fairbank type is more severe than the Ribbing type and is characterized by shortness of stature; short, stubby fingers; and small epiphyses in several joints, including the knee, ankle, hand, and hip (Fairbank 1947; Silverman 1996). The Ribbing type (Ribbing 1937; Silverman 1996) is confined predominantly to the hip joints and is characterized by hands that are normal and stature that is normal or near-normal. The unclassified types represent combinations of the Fairbank and Ribbing phenotypes.

Radiological examination of the skeleton shows delayed, irregular mineralization of the epiphyseal ossification centers and of the centers of the carpal and tarsal bones (Silverman 1996). Early-onset osteoarthritis (OA)

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is also a very common finding. Some patients with MED present with mild spinal and patellar abnormalities. The spinal changes can include irregular end plates of the vertebral bodies, Schmorl's nodes, wedging of the vertebral bodies, and narrowed disc spaces. Some patients may have ovoid vertebral bodies in the thoraco-lumbar spine in the first few years of life (Hulvey and Keats 1969; Silverman 1996). Radiographs of the knees show multipartite patellae in some MED cases (Sheffield 1998). The epiphyses are flat in the Ribbing type (Ribbing 1937) and are small in the Fairbank type (Silverman 1996).

MED is an autosomal dominantly inherited osteochondrodysplasia for which four loci have been identified so far: *EDM1* (MIM 132400), *EDM2* (MIM 600204), *EDM3* (MIM 600969), and *EDM5* (Chapman et al. 2001). *EDM1* is located on chromosome 19p13.1 (Newton et al. 1994) and contains a gene for cartilage oligomeric matrix protein (*COMP* [MIM 600310]) (Oehlmann et al. 1994). Eleven mutations in *COMP* have been identified in the Ribbing, Fairbanks, and unclassified forms of the disease (Briggs et al. 1995, 1998; Ballo et al. 1997; Susic et al. 1997; Ikegawa et al. 1998; Loughlin et al. 1998; Deere et al. 1999; Délot et al. 1999), and ~40 mutations have been identified in pseudoachondroplasia (PSACH [MIM 177170]), an allelic disorder (Deere et al. 1998, 1999; Ikegawa et al. 1998; Délot et al. 1999; Newman et al. 2000). The other two MED loci contain genes for collagen IX—namely, *COL9A2* (MIM 120260) on chromosome 1p32.3-p33 (Warman et al. 1994) and *COL9A3* (MIM 120270) on chromosome 20q13.3 (Brewton et al. 1995). Four mutations have been identified in the *COL9A2* gene (Muragaki et al. 1996; Holden et al. 1999; Spayde et al. 2000), and three have been identified in the *COL9A3* gene (Paassilta et al. 1999*a;* Bonneman et al. 2000; Lohiniva et al. 2000). Recently, Chapman and others (2001) identified a new MED locus, *EDM5*. It is located on chromosome 2p24-p23 and contains a gene for matrilin-3 (*MATN3* [MIM 602109]). Two different missense mutations were reported in the exon encoding the von Willebrand factor A domain of matrilin-3 in two unrelated families with MED (Chapman et al. 2001).

COMP is an extracellular matrix glycoprotein of 524 kD that belongs to the thrombospondin protein family (Hedbom et al. 1992; Mörgelin et al. 1992; Oldberg et al. 1992). It is a bouquet-shaped pentameric molecule composed of five identical arms (Mörgelin et al. 1992; Malashkevich et al. 1996), each containing a coiled-coil N-terminal domain responsible for pentamerization, four epidermal growth factor–like repeats, eight thrombospondin type 3 (T3) repeats, and a large globular C-terminal domain (Mörgelin et al. 1992; Hummel et al. 1998). Most of the mutations characterized in COMP in patients with MED or PSACH have been amino acid substitutions clustered in the T3 repeats (Briggs et al. 1995). It has been shown that such mutations may disturb calcium binding (Misenheimer and Mosher 1995; Chen et al. 2000; Maddox et al. 2000; Thur et al. 2001).

Collagen IX is a heterotrimer of α1(IX), α2(IX), and α3(IX) chains encoded by the *COL9A1* (MIM 120210), *COL9A2,* and *COL9A3* genes (Pihlajamaa et al. 1998; Paassilta et al. 1999*b*). It is a fibril-associated collagen with interrupted triple helices, consisting of three collagenous (COL1–COL3) and four non-collagenous (NC1–NC4) domains. It is a quantitatively minor cartilage component that is covalently cross-linked to collagen II fibrils via the COL2 domain (Diab et al. 1996). The NC3 domain functions as a hinge, allowing the other two more N-terminal domains, COL3 and NC4, to project away from the fibril surface. It has been suggested that these domains may mediate interactions between collagen IX and other matrix molecules. The three genes are almost identical in their genomic organization, with one exception—the *COL9A1* gene contains 38 exons, whereas the others have 32.

Some families with MED have been reported to have autosomal recessive MED (EDM4 [MIM 226900]). A homozygous R279W mutation was found in the diastrophic dysplasia sulfate transporter gene (*DTDST* [MIM 222600]) in one individual with autosomal recessive MED characterized by club foot and double layered patella (Superti-Furga et al. 1999). The same homozygous mutation was recently found in two unrelated sibships with apparently isolated club foot (Huber et al. 2001).

Here we studied 41 probands with MED, of whom 16 were familial. Recombinations were observed between *COMP, COL9A1, COL9A2,* and *COL9A3* and the phenotype in two families and between three of the genes and the phenotype in another two families. Since mutations could be identified in only 6 of 39 probands, we suggest that at least one additional locus must exist for MED.

Subjects and Methods

Subjects

Forty unrelated probands with MED (probands 1–40, table 1) were examined in the Departments of Orthopaedics and Medical Genetics at the University of Medical Sciences, Poznan, Poland, and in the Department of Orthopaedics at the University of Medical Sciences, Szczecin, Poland, and one proband (proband 41, table 1) was examined at the Department of Rheumatology, St. Thomas' Hospital, London (proband 41, table 1). A detailed clinical and radiological examination was performed on all the probands and on most of the affected family members. Sixteen of the probands had a positive family history of disease, and 25 had no family history.

Table 1

Clinical and Radiological Findings in the Probands

Proband (Sex)	Age at Onset (years)	Current Age (years)	Height (cm)	Joint Pain	Clinical Findings: Joint Limitation	Clinical Findings: Other	Radiological Findings: Epiphyses: Flat	Epiphyses: Small	Epiphyses: Irregular	Radiological Findings: OA	Radiological Findings: Other	Mutation
1 (F)	.3	18	172	H	H		H, K, S, W			H		
2 (F)	7	50	158	H	E, H, K	HA[a], K[b,c]	A, K		H	A, H, K, W		D420A[d]
3 (F)	7	42	163	K	H	HA[a]	E, H, K			H, K	P[e,f], Th[g]	R279W[h]
4 (M)	11	40	185	K		K[b], F[i]	K, W		K, W	K		
5 (F)	7	22	160	H	H, K	K[c]	K, S	H	A, H	H, K	P[f]	
6 (M)	9	18	174	H			H					
7 (F)	7	23	170	H			A, HA, H, W		H	H		
8 (M)	6	22	176	H, SP	H		A, H		A, H		Th[g]	P276R[d]
9 (F)	2	17	162	K		F[a,i], HA[j], K[c]	A, H		K	K	Th[g], P[k]	
10 (M)	11	17	168	H, K	H	HA[a], K[c]	A, E, H, K, W					T585M[d]
11 (M)	12	31	170	E, H, K	E, H	K[c]	E, K	H	E, H			
12 (M)	2	14	163	K		K[b]	H, K		A, H		Th-L[l]	
13 (M)	10	24	182	H	H		H		H			
14 (M)	3	41	160	H	E, H		E, HA, H, K, S		H, K	H	P[f], Th-L[g]	
15 (M)	6	20	182	K	H		A, H		A, H			
16 (M)	12	41	174	H, K	H		A, H		H	A, H		
17 (M)	1	3	90		H, K	F[i], K[b]	H, K					
18 (F)	7	41	152	A, H, K	H	HA[a]	A, E, HA, H, K		K	A	P[f], Th-L[g]	
19 (M)	5	42	141	H	H	K[b]	S		H			
20 (M)	0	20	158			HA[a]	HA	S, W	A		L[g]	
21 (F)	6	19	146	K			A	K, W	K			
22 (M)	5	37	164	K, H		F[g], H[b]	A, H, K		K		P[k], L[l]	
23 (M)	.3	26	172		H, K	F[a], HA[a], K[b]	E	H	E, H, HA, K, S, W			
24 (F)	13	22	160	H	H		H		H			
25 (F)	7	25	152		H, S, W	HA[a], K[b]	H, S				Th[g,l]	
26 (M)	1	23	171	H	H, K		H		A, E, H, K	E, H, K	P[f]	
27 (M)	2	16	180			K[b]	A, H, K		K		P[k]	
28 (M)	2.5	6	115	H	H		HA, K	H	H			
29 (F)	2	4	108		K	H[b]		H, K				
30 (F)	1	9	129			H[b]	K		H			
31 (M)	12	19	168		H	E[b]	E, H, K					
32 (M)	4.5	16	161	K, H	H		H					
33 (F)	7	19	163	H	H		H		H			
34 (F)	1	23	157	K, H		F[a], HA[a]		H	H, W	H		
35 (F)	1	12	150	H	H	E[j], HA[j]	H					
36 (F)	3	8	122		H, K	K[b]	H, K				Th[g], P[f]	R279W[h]
37 (F)	4	8	122	K		H[b]	A, K				P[k]	
38 (M)	5	12	130	H				H	H, K		Th-L[g]	
39 (M)	12	31	162	H	H, K, S	F[i]	A, H, S		E, HA, K	H, K		
40 (M)	9	12	150	K		H[b], K[b]	H		K		Th-L[g]	
41 (M)	10	30	180	K, H					K, Th-L		Th-L[g]	InsT^{+3}IVS8[m]

NOTE.—A = ankles; E = elbows; F = feet; H = hips; HA = hands; K = knees; L = lumbar vertebral bodies; P = patella; S = shoulders; SP = spine; Th = thoracic vertebral bodies; W = wrists.

[a] Brachydactyly.
[b] Valgus/varus deformity.
[c] Crepitation.
[d] In the *COMP* gene.
[e] Lateral dislocation.
[f] Multipartite patella.
[g] Endplate irregularities.
[h] In the *DTDST* gene.
[i] Talipes deformities.
[j] Joint laxity.
[k] Developmental delay.
[l] Mild platyspondyly.
[m] In the *COL9A1* gene.

Blood samples were obtained from the probands and the family members, for genomic DNA isolation. Signed informed consent was obtained from all subjects.

Linkage Analysis

Intragenic markers or microsatellite markers were used for linkage analysis of *COL9A1, COL9A2, COL9A3,* and *COMP*. Three sequence variations were tested in *COL9A1:* T→C in IVS2^{-12}, C→T in IVS19^{-88}, and A→G in IVS20^{+61}. The IVS2 variation was amplified by PCR with the primers E3F (5′-GTG GTC AAT TGC TAT TTT CTG GTT C) and E3R (5′-GCT TTA TCT ACC TGG AAC TGA G), and the IVS19 and IVS20 variations were amplified with the primers E20F (5′-CCA TCA GAA GAA TTC TCC TTG GAC) and E20R (5′-GAT AAA AAG TTA TGT TTA AAT GGC). Primers A19F (5′-TGG ATC TCA GTT TCC CTA CCT G) and A19R (5′-CAA GAG GTG GTG ATT GAG CAA GAG C) were used for PCR amplification of a region of the *COL9A2* gene that contained three single-nucleotide variations: A→G in exon 19^{+23}, C→G in exon 19^{+49}, and C→G in IVS18^{-4}. In addition, microsatellite markers, *DIS211* and *L-MYC*, were used to analyze linkage to chromosome 1 (Mäkelä et al. 1992; Weissenbach et al. 1992). The primer pair FI19C (5′-CAG AAT GGC GTG CCA GGA CTC G) and RI19C (5′-CCA ACA TGG GCC ACT GAG C) was used to amplify four single-nucleotide variations in the *COL9A3* gene by PCR; G→C in IVS18^{-90}, C→T in IVS19^{+10}, G→C in IVS19^{+12}, and G→A in IVS19^{+58}. The primer pair JC18F (5′-GGG CTG GCC ACT GAA GCT CTG AGA) and JC18R (GCC GCG GTG AGG GTG GCT GCT AT) was used to amplify a single-nucleotide variation in the *COMP* gene, T→C in IVS18^{+53}. A tetranucleotide repeat in IVS9 of the *COMP* gene had been reported elsewhere (Briggs et al. 1995).

Mutation Screening and Sequencing

Exons and the boundaries of the *COMP* (Newton et al. 1994; GenBank accession number AC003107), *COL9A1, COL9A2* (Pihlajamaa et al. 1998), and *COL9A3* (Paassilta et al. 1999*b*) genes were amplified by PCR using 40 ng of genomic DNA, 0.25 μM of forward and reverse primers, 1.5 μM $MgCl_2$, 0.2 mM dNTPs, and 1 U of Ampli*Taq* Gold polymerase (Perkin Elmer). The PCR conditions included an initial denaturation for 10 min at 95°C, followed by 35 cycles at 95°C for 30 s, at 54°C–63°C for 30 s, and at 72°C for 30 s, followed by 1 cycle at 72°C for 10 min. This was followed by denaturation at 98°C for 3 min and reannealing at 68°C for 30 min, to generate heteroduplexes. All PCR products were checked for quality and quantity on 2% agarose gels.

Conformation-sensitive gel electrophoresis (CSGE) was performed as described elsewhere (Körkkö et al. 1998), and the PCR products that contained heteroduplexes were sequenced using the dRhodamine Terminator Cycle Sequencing Ready Reaction Kit and an ABI Prism 377 Sequencer (Perkin Elmer), after prior treatment with exonuclease and shrimp alkaline phosphatase (Werle et al. 1994).

A region of the *DTDST* gene containing the R279W mutation was amplified by PCR with a primer pair that has been described elsewhere (Huber et al. 2001). The presence of the mutation was studied by digestion with *Sty*I restriction endonuclease.

RNA Analysis

Total RNA was extracted from Epstein-Barr virus (EBV)–transformed lymphoblasts from proband 41. First-strand cDNA synthesis (Superscript Preamplification System [Gibco BRL]) was followed by PCR amplification with primers corresponding to exon 5 (D1F: 5′-GCA GCC TTT TCG AAT TTG TCC TCC TTG) and exon 19 (D1R: 5′-CTC CGA GTT CTC CCT GGT CAC CTT CTT CAC) of the *COL9A1* gene. A second amplification was performed using nested primers from exon 6 (JV-9B: 5′-GAA ACT TGC CAT GAG CTG CCA) and exon 11 (JV-8R: 5′-ATC CAT CAG GTC CTG TTA AT). The products were analyzed on agarose gels and by sequencing.

Results

Diagnosis

Initially, 59 probands had been selected for examination, on the basis of their clinical histories and clinical findings, which were typical of MED. Radiographs were taken of the ankles, elbows, hands, hips, knees, shoulders, and spine and were evaluated independently by two expert radiologists. After evaluation of the clinical and radiological findings, a diagnosis of MED was confirmed in 41 cases; the remaining eighteen probands were excluded because they were found to have bilateral Legg-Calve-Perthes disease (MIM 150600), spondyloepiphyseal dysplasia tarda (MIM 184100), spondyloepimetaphyseal dysplasia (MIM 300106) or mild to severe forms of PSACH. The detailed clinical and radiological findings regarding the probands are presented in table 1, and radiographs of the knees and spine of proband 14 and of the hips of the proband's affected brother are shown in figure 1*A–E*.

Linkage Analysis

Of the cases of disease in the 41 probands, 25 were sporadic and 16 were familial. Linkage to all four candidate genes, *COL9A1, COL9A2, COL9A3* and *COMP,* was tested in the four largest families available (probands 12, 14, 20, and 32; table 1), yielding recombinations with

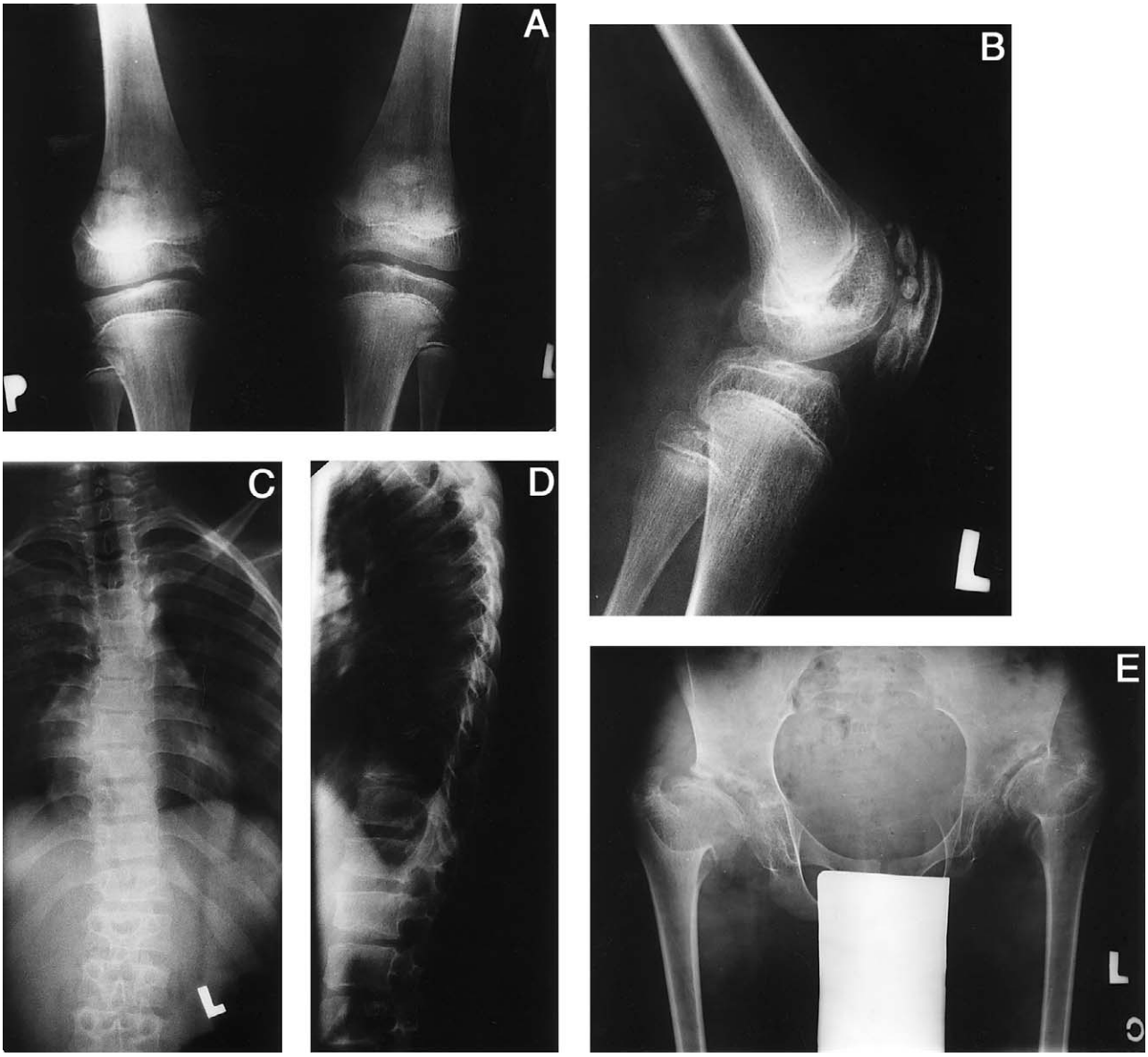

Figure 1 Radiographs of the knees (*A,* anteroposterior (AP) view; *B,* lateral view) and thoraco-lumbar spine (*C,* AP view; *D,* lateral view) of proband 14 at age 14 years (table 1), and of the hips (*E*) of the proband's affected brother at age 11 years (individual R.B. in fig. 2). The knee radiographs show irregular and flat epiphyses and multipartite patella. Endplate irregularities are seen in the thoraco-lumbar spine. The radiographs of the hips show varus deformity, shortening of the femoral necks, enlarged and flat femoral heads, dysplastic acetabuli, and dislocation of the left hip.

the MED phenotype in two of the families, and recombinations between *COL9A2, COL9A3* and*COMP* and the phenotype in the other two (fig. 2).

Mutations and Polymorphisms in the COMP *Gene*

All probands except for numbers 12, 14, 20, and 32 (table 1 and fig. 2) were analyzed for mutations in *COMP*. All 19 exons and exon boundaries of the gene were amplified by PCR and were tested for sequence variations by CSGE. Products that contained heteroduplexes in CSGE analyses were sequenced, leading to the identification of three mutations (fig. 3). The rest of the observed sequence variations were likely to be neutral, because they did not change the amino acid encoded and were also found in controls (not shown).

A novel A→C transversion was found in exon 12, which changed a GAT codon for Asp^{420} to a GCT codon for Ala in the sixth T3 repeat (fig. 3). This was found in proband 2 (table 1) and in her two affected daughters, who were aged 16 and 20 years, but not in any unaffected family members or in the 100 controls tested. The younger daughter had had knee pains since the age 7

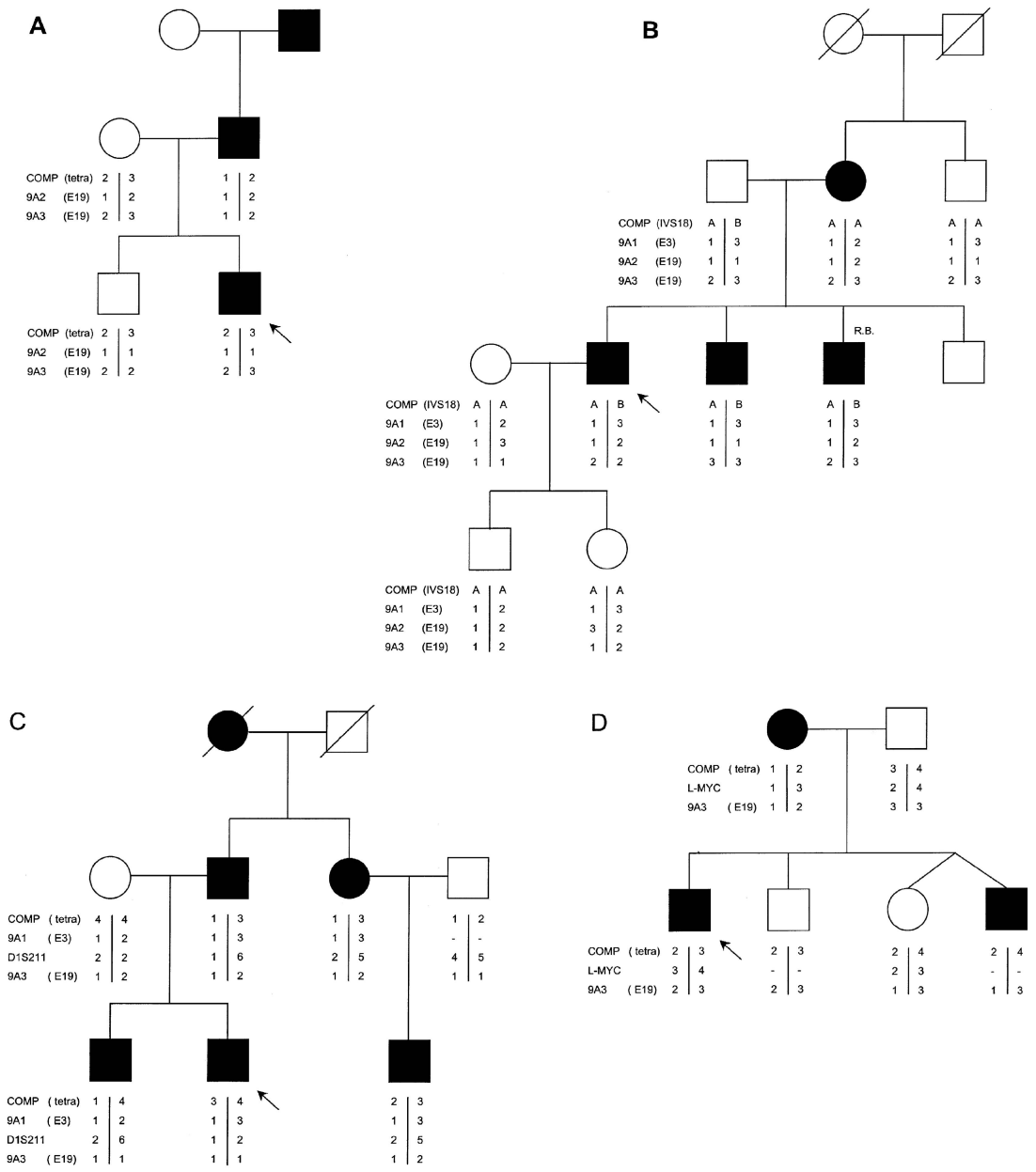

Figure 2 Pedigrees of four families with MED. Blackened symbols denote affected individuals. The probands are indicated by arrows (↖). The family pedigree of proband 12 is shown in panel *A*, that of proband 14 in panel *B*, that of proband 20 in panel *C*, and that of proband 32 in panel *D* (table 1). Alleles of the markers are shown below the symbols.

years. Clinical examination revealed crepitation of the knees, laxity of the knee joints, and mild scoliosis, while radiological examination showed subchondral sclerosis, flattening of the femoral heads, valgus deformity, short femoral necks, and irregular and flat epiphyses of the knees (not shown). Arthroscopy revealed a bipartite patella and chondromalacia patellae. Her older sister had had hip pains since age 14 years and currently had knee and elbow pains as well. A clinical examination revealed valgus deformity of the forearms, hyperextension of the elbows, joint laxity, crepitation of the knees, mild scoliosis, and mild shortening of the toes. Radiographs showed valgus deformity of the femoral neck, mild flattening of the femoral heads, irregular and flat knee epiphyses, sclerosis of the acetabulum, and endplate irregularities of the lumbar vertebral bodies (not shown).

A second mutation, identified in proband 8 (table 1), consisted of a single-base change in exon 8 (fig. 3) that converted a CCG codon for Pro^{276} to a CGG codon for Arg in the first T3 repeat. Four other family members

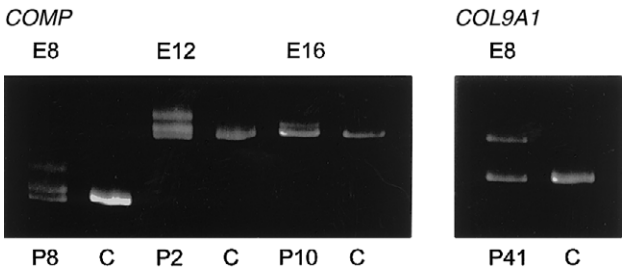

Figure 3 CSGE analysis of the PCR products for exons 8, 12, and 16 of the *COMP* gene, and for exon 8 of the *COL9A1* gene. "E" denotes exon, "P" denotes proband, and "C" denotes control.

were also affected: the proband's mother and maternal grandmother, an aunt, and an uncle. All of them had the mutation, which was not found in any of the unaffected family members. The mutation was not found in any of the 100 controls tested. The proband's mother is 46 years old and 168 cm tall. She has had difficulties in walking since age 15 years and had hip pain since age 23 years. At the time of the present study, she also had pain in the knees, shoulders, and wrists. Clinical examination revealed crepitation of the shoulders, valgus deformity of the knees, and a limited range of motion of the hips. Radiological examination showed severe hip and knee OA, flattening of the femoral heads and knee epiphyses, shortening of the femoral neck, and endplate irregularities in the lumbar spine (not shown). The uncle is 44 years old and 172 cm tall. He has had a waddling gait and hip pain since age 3 years and has a limited range of movement in his hips and knees. Radiological examination revealed flattening of the knee epiphyses with moderately severe OA, flat femoral heads with multiple subchondral cysts, varus deformity of the hips, and short femoral necks (not shown). A diagnosis of MED was confirmed in the other two affected individuals by clinical and radiological examination.

The third mutation, affecting exon 16 in proband 10 (table 1, fig. 3), changed an ACG codon for Thr^{585} to an ATG codon for Met in the C-terminal globular domain. None of the proband's family members were affected, nor did they carry the mutation, suggesting a de novo mutation. The proband had had knee pain since age 6 years and hip pain since age 12 years. The clinical and radiological findings are presented in table 1. There was no radiological evidence of metaphyseal or vertebral-body abnormalities.

Mutation Analysis of the COL9A1, COL9A2, *and* COL9A3 *Genes*

All the probands in whom no mutations were found or no recombinations were observed between the collagen IX genes and the phenotype were analyzed for sequence variations in the genes. Exons and the boundaries of the *COL9A1, COL9A2,* and *COL9A3* genes were amplified by PCR and were analyzed for heteroduplexes by CSGE. In addition, exons 2, 3, and 4 of the *COL9A2* and *COL9A3* genes and exons 8, 9, and 10 of the *COL9A1* gene were analyzed by sequencing.

Analysis of exon 8 of the *COL9A1* gene in proband 41 identified a unique heteroduplex (table 1, fig. 3). Sequencing of the product showed insertion of a T at the donor splice site of $IVS8^{+3}$ (data not shown). The presence of the insertion was confirmed by digestion with *Mse*I restriction endonuclease (data not shown). The proband's affected mother also had the insertion, whereas his unaffected sister did not. The insertion was not found in any of 600 control chromosomes tested. RNA isolated from the lymphoblasts of the affected son and from a control sample was reverse transcribed and amplified by PCR, using primers specific to exons 6 and 11. The products were first analyzed on agarose gels, where the control sample always had only one band, but the sample from the affected individual constantly showed two major bands and one or more minor ones. One of the major bands corresponded to the control band in size, whereas the other one was ~150 bp shorter (fig. 4). Sequencing of the PCR products indicated that the insertion resulted in at least three splicing defects (not shown): one lacking sequences for exons 8 and 10, and the other two lacking sequences for either exon 8 or exon 10. Skipping of exon 8, exon 10, or exons 8 and 10 leads to an in-frame deletion of 25, 21, or 46 amino acids, respectively, in the COL3 domain of the α1(IX) chain.

The proband, now aged 30 years, has had knee pains

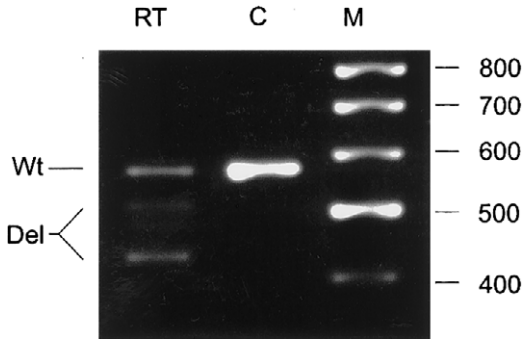

Figure 4 Agarose gel electrophoresis of α1(IX) reverse-transcriptase PCR products. RNA was isolated from EBV-transformed lymphoblasts from proband 41 and was analyzed for splicing by PCR as indicated in the Subject and Methods section. The control sample (C) shows one product of about 570 bp, whereas the sample from the proband (RT) has two major products of about 570 and 430 bp. In addition, two small minor products can be seen in the proband's sample. "Wt" denotes wild type, "Del" denotes deletion, and "M" denotes molecular weight marker.

and difficulty in walking since the age of 10 years, and pain and stiffness in the knees had persisted. Radiographs indicated evidence of early OA in the right knee, Schmorl's nodes, endplate irregularities, and anterior osteophytes in the thoraco-lumbar vertebrae, including calcification of the intervertebral disc (fig. 5*A* and *B*). The hips were normal, but the sacro-iliac joints were ill defined. The proband's mother had arthritis affecting her hands, feet (fig. 5*C*), hips (fig. 5*D*), knees (fig. 5*E*), and spine, with symptoms starting at age 45 years. She has had a total knee replacement. The proband's sister was asymptomatic, but their maternal grandmother had bilateral hip replacements.

Several sequence variations were identified, although these were likely to be neutral, since they were also found in controls. The variations that resulted in amino acid changes are shown in table 2. One of the variations in *COL9A3*, G→A resulting in the conversion of Arg to Gln, was not found in any of the 109 controls tested. This variation was found in proband 21 and in her unaffected father. Thus, the variation most likely represents a rare neutral sequence variant.

Analysis of the DTDST *gene*

Because a homozygous R279W *DTDST* mutation has been reported in an individual with autosomal recessively inherited MED characterized by club foot and/or

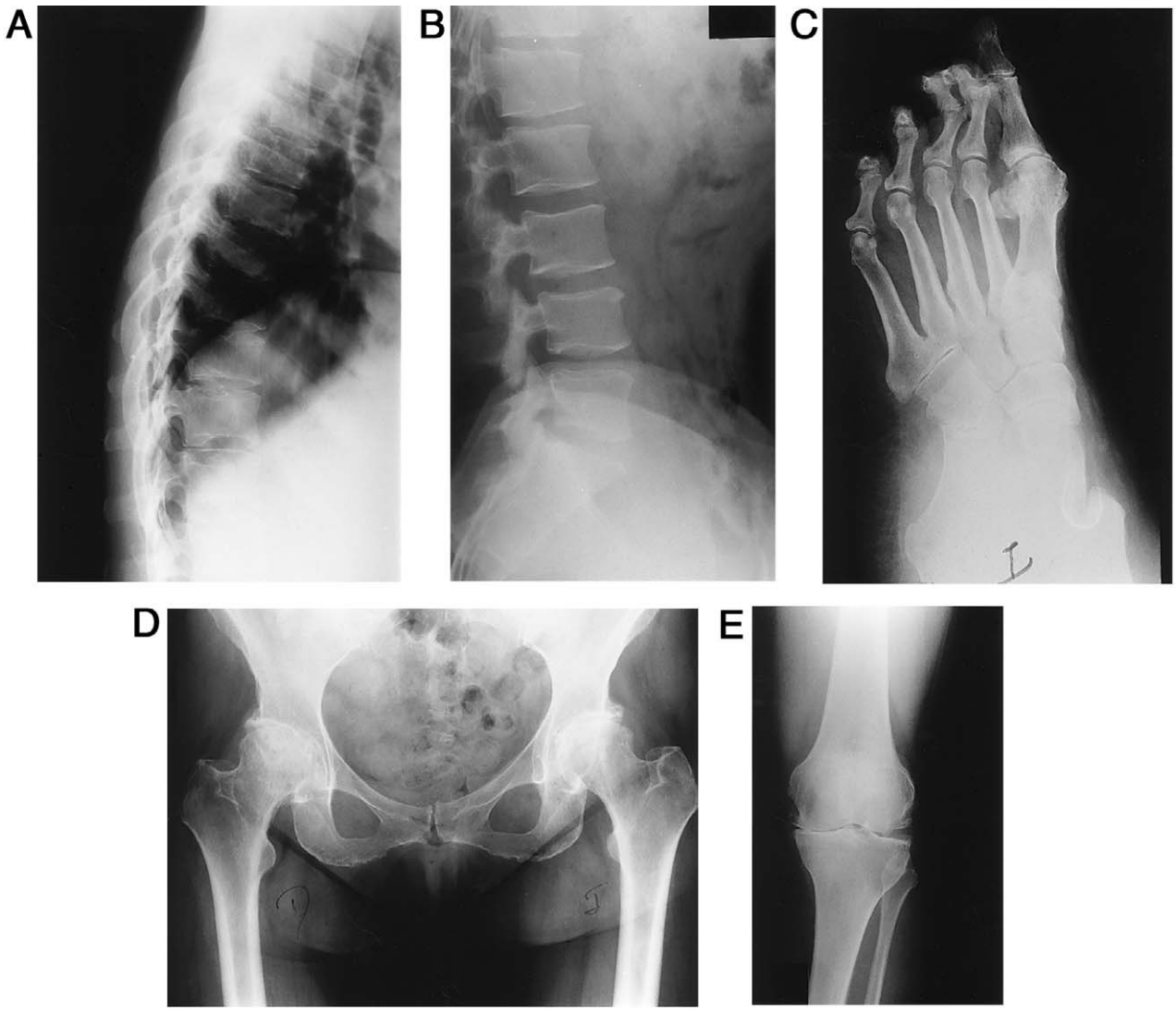

Figure 5 Radiographs of the thoraco-lumbar spine (*A,* lateral view) and lumbar spine (*B,* lateral view) of proband 41 (table 1), and of the left foot (*C,* AP view), hips (*D,* AP view), and left knee (*E,* AP view) of the proband's affected mother. Radiographs of the spine show small anterior osteophytes on several of the lower thoracic vertebrae and on L4. Radiographs of the proband's mother show widespread and severe OA in all joints.

Table 2

Amino Acid Variations in *COL9A1, COL9A2,* and *COL9A3*

Gene and Exon	Nucleotide Change	Amino Acid Change	Allele Counts[a]	
			Probands[b]	Controls[c]
COL9A1:				
11	1066T→C	S339P	2/68	1/99
28	1913G→A	R621Q	8/62	21/79
COL9A2:				
14	780C→T	T246M	4/66	2/98
19	1020A→G	Q326R	12/58	23/93
19	1046C→G	L335V	2/68	5/111
COL9A3:				
5	324C→T	R103W	5/65	7/93
5	325G→A	R103Q	1/69	1/99
17	904C→T	P296L	2/68	1/99
23	1222G→A	R402Q	1/69	0/218
25	1321C→A	A435E	12/58	22/78

[a] Allele count to the left of the slash is that of the polymorphic allele, and the allele count to the right of the slash is that of the wild-type allele. For instance, in the case of *COL9A1* exon 11, 2 alleles were 1066T and 68 alleles were 1066C in the probands.

[b] Allele counts were determined from 35 probands. Allele counts were not determined from probands 2, 8, 10, 14, 32, and 41.

[c] Allele counts were determined from ⩾50 control subjects.

double-layered patella, we tested for the presence of the mutation in the probands who had either multipartite patella or talipes deformities (probands 3, 4, 5, 14, 17, 18, 22, 26, 36, and 39; table 1) by *Sty*I restriction-enzyme digestion. No heterozygotes were found, but probands 3 and 36 were homozygous for the mutation (data not shown). The mutation was confirmed by sequencing (data not shown). The parents of probands 3 and 36 were unaffected. Samples from the parents were not available for mutation analysis.

Discussion

MED is, clinically and radiologically, a highly heterogeneous disease for which five loci have so far been identified. Eleven mutations in *COMP* have been found in the Ribbing, Fairbanks, and unclassified forms (Briggs et al. 1995, 1998; Ballo et al. 1997; Susic et al. 1997; Ikegawa et al. 1998; Loughlin et al. 1998; Deere et al. 1999; Délot et al. 1999), and all of these mutations have been different and have been located in different domains of the molecule, thus probably partially explaining the clinical heterogeneity. However, all of the mutations identified to date in the other two genes, *COL9A2* and *COL9A3,* have had the same consequence—that is, skipping of exon 3 and a resulting in-frame deletion of 12 amino acids (Muragaki et al. 1996; Holden et al. 1999; Paassilta et al. 1999*a;* Bonneman et al. 2000; Lohiniva et al. 2000; Spayde et al. 2000). All individuals with collagen IX splicing mutations also share a very similar phenotype, typically consisting of normal to near-normal height, as well as epiphyseal dysplasia of the knees and some other joints in childhood, but generally only OA of the knees in adulthood.

The phenotypic heterogeneity of MED may also be due to locus heterogeneity, a hypothesis supported by the results of the present linkage analysis. The analysis of four families with MED showed recombinations between the four candidate genes and the phenotype in two of the families, and between three of the candidate genes and the phenotype in the other two families. The results of the mutation screening analysis further supported the possibility of locus heterogeneity. Surprisingly, screening of the exon sequences and exon boundaries for the mutations in the four candidate genes, *COL9A1, COL9A2, COL9A3,* and *COMP,* resulted in the identification of only four mutations in the probands, a finding that suggests that mutations in collagen IX and COMP do not explain the majority of MED cases. Even though the CSGE method has proved to be highly sensitive (Körkkö et al. 1998), it is possible that some mutations have gone undetected. One of the limitations of the method is that it does not detect large deletions, and it is true that only point mutations or small deletions have been reported in the genes for collagen IX and COMP in patients with MED.

Of the three mutations found in *COMP,* two were novel, and both were located in the T3 repeats, as has been the case with most of the previously characterized mutations (Briggs et al. 1995, 1998; Ballo et al. 1997; Susic et al. 1997; Ikegawa et al. 1998; Loughlin et al. 1998; Deere et al. 1999). The third mutation, Thr^{585} to Met in the C-terminal globular domain, has been detected previously in a family with a mild form of PSACH (Briggs et al. 1998). The finding that the same mutation can lead to different but overlapping phenotypes is not surprising. First, the various MED and PSACH forms make up a phenotypic spectrum, and second, there are several examples of the same mutations causing variable phenotypes in different individuals—for example, collagen I mutations in osteogenesis imperfecta (MIM 166210 and MIM 166200) (Körkkö et al. 1997). Phenotypic variation can be evident even among members of one family, as was the case in one family with MED family that had a *COL9A3* mutation (Paassilta et al. 1999*a*).

The mutation in *COL9A1* identified in one family represents the first human disease-causing mutation ever reported in connection with this gene. Since *COL9A1* contains six additional exons coding for the longer NC4 domain compared with the two other collagen IX genes, exon 9 of *COL9A1* corresponds to exon 3 in the others. For this reason, it was somewhat surprising that the mutation, the insertion of a T at the donor splice site of $IVS8^{+3}$, did not result in skipping of exon 9. The finding that it led to a complex splice pattern involving mainly

exons 8 and 10 was not in itself surprising, however, since mutations at donor splice sites have been shown to lead to variable splice forms (Schwarze et al. 1999).

Although the exact phenotype of the two individuals carrying the *COL9A1* splicing mutation is unclear in terms of whether cartilage degeneration typical of primary OA or of chondrodysplasia is the predominant feature, the phenotype of MED in the proband is supported by a history of knee pain and walking difficulties in childhood, as well as by the nature of the mutation. It has been shown elsewhere that a diagnosis of MED is difficult to establish in adult patients, in the case of collagen IX mutations (Muragaki et al. 1996; Holden et al. 1999; Paassilta et al. 1999*a;* Bonneman et al. 2000; Lohiniva et al. 2000; Spayde et al. 2000). Consequently, these mutations should be considered when evaluating patients with familial OA primarily affecting the knees.

Two additional loci, *EDM4* and *EDM5,* have been identified recently in patients with MED. *EDM4* contains the *DTDST* gene, in which a homozygous R279W mutation was shown to cause recessively inherited MED associated with club foot and double-layered patella (Superti-Furga et al. 1999). The same homozygous mutation was also reported in two sibships with apparently isolated club foot (Huber et al. 2001). This mutation was detected here in two probands with multipartite patella. The *EDM5* locus contains the *MATN3* gene (Chapman et al. 2001). Two different mutations were reported very recently in this gene in two families with autosomal dominant MED (Chapman et al. 2001). Since this gene was not analyzed here, it is possible that some of the probands in the present study may have mutations in this gene.

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Accession numbers and the 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], *EDM1* [MIM 132400], *EDM2* [MIM 600204], *EDM3* [MIM 600969], *EDM4* [MIM 226900], *COMP* [MIM 600310], PSACH [MIM 177170], *COL9A1* [MIM 120210], *COL9A2* [MIM 120260], *COL9A3* [MIM 120270], *DTDST* [MIM 222600], *MATN-3* [MIM 602109], Legg-Calve-Perthes disease [MIM 150600], spondyloepiphyseal dysplasia tarda [MIM 184100], spondyloepimetaphyseal dysplasia [MIM 300106], and osteogenesis imperfecta [MIM 166210 and MIM 166200])

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