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Identification of Novel pro-α2(IX) Collagen Gene Mutations in Two Families with Distinctive Oligo-Epiphyseal Forms of Multiple Epiphyseal Dysplasia

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Summary

Multiple epiphyseal dysplasia (MED) is a genetically heterogeneous disorder with marked clinical and radiographic variability. Traditionally, the mild "Ribbing" and severe "Fairbank" types have been used to define a broad phenotypic spectrum. Mutations in the gene encoding cartilage oligomeric-matrix protein have been shown to result in several types of MED, whereas mutations in the gene encoding the α2 chain of type IX collagen (*COL9A2*) have so far been found only in two families with the Fairbank type of MED. Type IX collagen is a heterotrimer of pro-α chains derived from three distinct genes—*COL9A1, COL9A2,* and *COL9A3*. In this article, we describe two families with distinctive oligo-epiphyseal forms of MED, which are heterozygous for different mutations in the *COL9A2* exon 3/intron 3 splice-donor site. Both of these mutations result in the skipping of exon 3 from *COL9A2* mRNA, but the position of the mutation in the splice-donor site determines the stability of the mRNA produced from the mutant *COL9A2* allele.

Introduction

Multiple epiphyseal dysplasia (MED; MIM 226900) is a relatively common osteochondrodysplasia resulting in mild short stature and early-onset osteoarthritis (International Working Group on Constitutional Diseases of Bone 1998). MED is clinically and radiographically heterogeneous, and, typically, the mild "Ribbing" (Ribbing 1937) and severe "Fairbank" (Fairbank 1947) types are used to define a broad phenotypic spectrum that also includes a number of unclassified types of MED. Individuals with MED are usually diagnosed in mid to late childhood, because of pain and stiffness of the large joints, such as ankles, knees, or hips (Rimoin and Lachman 1993). In some types of MED, severe progressive osteoarthritis results in hip replacement as early as the 3d or 4th decade of life.

Sir Thomas Fairbank published, >50 years ago, the first detailed account of a disorder that he described as "dysplasia epiphysialis multiplex" (Fairbank 1947). This remarkable paper was the product of >35 years of clinical investigation and documented the clinical and radiographic findings on 20 patients with MED (Fairbank 1909, 1925, 1927, 1935, 1946). Although Fairbank concluded that MED, "as a rule, is not inherited or familial" (Fairbank 1947, p. 16), later reports clearly established that MED could be inherited as an autosomal dominant disorder (Waugh 1952; Maudsley 1955; Shephard 1956). In 1958, Barrie et al. reported a family with 11 affected individuals in four generations and suggested that at least two genes are responsible for MED—one for the relatively mild type seen in their family and another for the more severe type seen in other families.

Successive genetic-linkage and positional candidate–cloning studies have shown that mutations in at least three genes can result in phenotypes within the MED disease spectrum. Mutations in the gene encoding cartilage oligomeric-matrix protein (COMP), a pentameric glycoprotein localized to the territorial matrix surrounding chondrocytes, can result in Fairbank, Ribbing, and unclassified types of MED (EDM 1; MIM 132400) (Briggs et al. 1995, 1998; Ballo et al. 1997; Susic et al. 1997; Loughlin et al. 1998). Furthermore, it was also determined that mutations in the gene encoding the α2 chain of type IX collagen (*COL9A2*) can result in the

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Fairbank type of MED (EDM 2; MIM 600204) (Briggs et al. 1994; Muragaki et al. 1996; van Mourik et al. 1998*a*). Type IX collagen is a member of the FACIT (*f*ibril-*a*ssociated *c*ollagen with *i*nterrupted *t*riple-helix) group of collagens and is a minor component of the cartilage extracellular matrix (Olsen 1997). It is a heterotrimer, α1(IX)α2(IX)α3(IX), of polypeptides derived from three distinct genes (*COL9A1, COL9A2,* and *COL9A3*), and this has led us to the hypothesis that mutations in the *COL9A1* and *COL9A3* genes may also result in MED phenotypes. Preliminary evidence from linkage analysis has confirmed that there is at least a third MED disease locus (EDM 3; MIM 600969), but it has not been determined whether the *COL9A1* and *COL9A3* genes are involved (Deere et al. 1995; M. D. Briggs and G. R. Mortier, unpublished data).

Because of the recent discovery that a mutation in the intron 3 splice-donor site of *COL9A2* produces the Fairbank type of MED (Muragaki et al. 1996), we have screened affected individuals from 28 unrelated families with various types of MED, for mutations in the exon 9/intron 9 splice-donor site of *COL9A1* and in the exon 3/intron 3 splice-donor sites of *COL9A2* and *COL9A3*. These three exons encode equivalent segments of the α1(IX), α2(IX), and α3(IX) chains, respectively, and constitute part of the COL3 domain of type IX collagen. In the present study, we report the identification of novel mutations in the exon 3/intron 3 splice-donor site of *COL9A2* in two families with distinctive oligo-epiphyseal forms of MED. The position of the mutation in the splice-donor site determines the stability of the mRNA produced from the mutant *COL9A2* allele, which may be a contributing factor in both the pathology and the clinical variability of MED. The findings presented in this report confirm that MED can result from mutations in the *COL9A2* gene and support the evidence that MED shows considerable genetic heterogeneity.

Subjects and Methods

Clinical Summary

DNA was prepared from blood collected from both affected and unaffected individuals in 28 families with various types of MED. Informed consent was obtained prior to the collection of blood samples. Clinical geneticists and/or radiologists with extensive experience in the diagnosis of skeletal dysplasias have examined the clinical history and appropriate x-rays of the two families in which we identified a *COL9A2* mutation.

Family K.—The proband (JK) is a 17-year-old male of normal height (figs. 1*a* and 2*a*) who was diagnosed in childhood with pain in the joints (mostly the knees) and genu varum. Radiographs show major epiphyseal changes in the knees (fig. 2*b*) and hands (not shown), and almost no changes in the hips (fig. 2*c*). The spine (not shown) also appears normal.

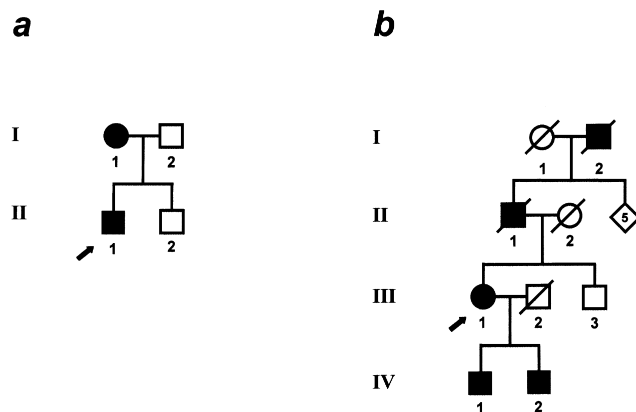

Figure 1 Pedigrees of two families with MED and *COL9A2* mutations. *a,* Family K. *b,* Family G. In both families, an arrow indicates the proband.

Family G.—Affected members of family G (fig. 1*b*) demonstrate evidence of widespread epiphyseal dysplasia affecting, particularly, the large joints of the lower limbs (fig. 2*d* and *e*). They are slightly short in stature (adult height is 158 cm), and all three affected individuals developed genu valgum, which required surgical correction. The proband, individual III-1 (MG), started walking late and with a stiff gait that became more noticeable during ages 5–7 years. Her legs became increasingly bowed, and at age 17 years she underwent tibial osteotomy. She has developed increasing problems with pain in her hands, lumbar spine, and ankles. Individuals IV-1 and IV-2 walked at age 2 years with a stiff gait. Epiphyseal dysplasia was recognized in them in early childhood, and they underwent tibial osteotomy for genu valgum at ages 14 and 13, respectively. They continue to have mild joint symptoms, including intermittent lumbar back pain and knee pain with swelling.

Mutational Analysis in Genomic DNA

Genomic DNA was amplified by PCR and was screened for mutations with a combination of SSCP and heteroduplex analysis, as described elsewhere (Briggs et al. 1995, 1998). In brief, PCR amplifications were performed in 100-μl reactions containing 1.5 mM $MgCl_2$, 10 mM Tris-HCl pH 8.3, 50 mM KCl, 200 μM of each dNTP, 10 pmol of each primer, 50–100 ng of genomic DNA, and 1 U of *Taq* DNA polymerase. PCR cycling consisted of 40 cycles of 94°C for 1 min, 55°–63°C for 1 min, and 72°C for 1 min. For SSCP and heteroduplex analysis, PCR products were resolved with 6% PAGE;

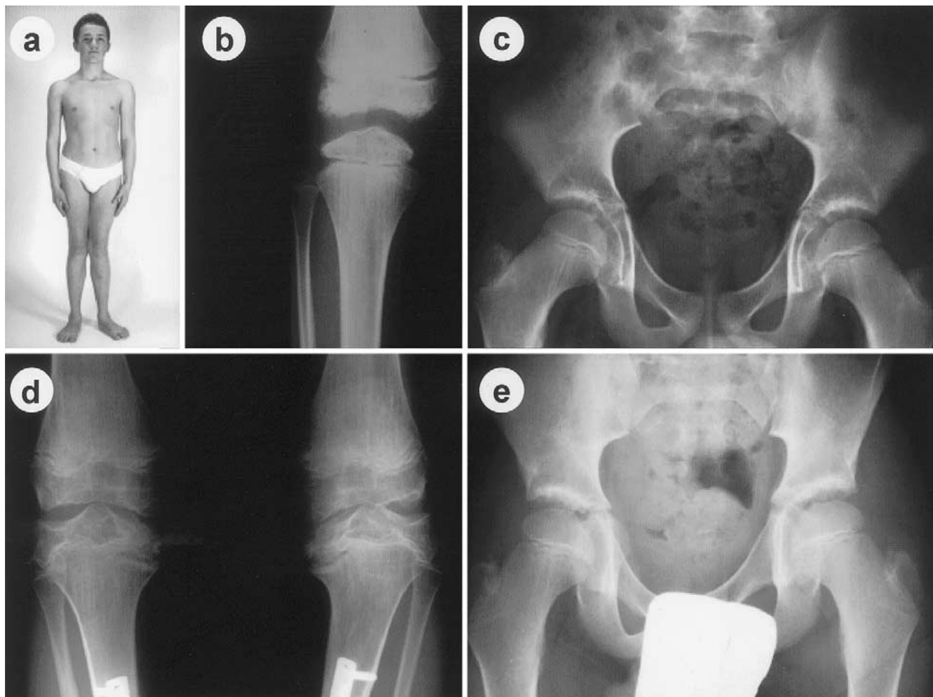

Figure 2 Radiographic findings in individual JK and affected individuals from family G. *a,* Individual JK at age 15 years. *b,* Individual JK at age 9 years 9 mo. Note flattened and irregular epiphyses. *c,* Individual JK at age 11 years 5 mo. *d,* Individual IV-2 (family G) at age 14 years 6 mo. Note flattened irregular epiphyses and metal plates from the tibial osteotomy that was performed at age 13 years. *e,* Individual IV-1 (family G) at age 10 years.

gels were precooled and were run at 4°C for 4–5 h at 300 V, and the DNA was visualized by silver staining.

To screen for mutations in the exon 3/intron 3 splice-donor site of *COL9A2,* DNA of the patient was amplified by PCR using forward (5′-caatgggccccctgaaaagct-3′) and reverse (5′-caatcccgggcttcccgtctg-3′) primers, located in exon 3 and intron 3, respectively (Muragaki et al. 1996). To analyze the exon 3/intron 3 splice-donor site of *COL9A3,* control genomic DNA was initially amplified by PCR, with the use of forward and reverse primers located in exons 3 and 4, respectively (H36F, 5′-cattgacggagaagctggtc-3′; and H44R, 5′ctctcctggtttccc-cggct-3′ [Brewton et al. 1995]). DNA sequence generated from this genomic fragment was then used to design a reverse primer that was specific to intron 3 of *COL9A3* (9A3in3R, 5′-gcaggatgagacaggatatca-3′). PCR with primers H36F and 9A3in3R amplified the exon3/intron3 splice-donor site, generating the expected DNA fragment of 140 bp. To amplify by PCR the equivalent region in *COL9A1,* we predicted the genomic structure of the COL3 domain of *COL9A1*, using the previously determined location of exons 1–8 (Muragaki et al. 1990) and 15–17 (McCormick et al. 1987) and the known structure of the mouse *col9a2* gene. Primers 9A1ex9F (5′-ggtga-ccgaggtcctaagg-3′) and 9A1ex10R (5′-agctcctggctttcccg-gtt-3′), which were predicted to lie in exons 9 and 10, respectively, amplified a genomic fragment of 199 bp. The location of these primers was later confirmed by the publication of the *COL9A1* genomic structure (Pihlajamaa et al. 1998).

Analysis of COL9A2 *cDNA*

RNA isolation was performed by means of TRIzol (Gibco BRL) and cDNA, synthesized with Superscript (Gibco BRL) according to the manufacturer's protocol. *COL9A2* cDNA was amplified by PCR with primers 9A2ex2F (5′-agaggtccaccgggagagcgg-3′) and 9A2ex5R (5′-ccccccttggctccagttaaac-3′), located in exons 2 and 5, respectively.

DNA sequencing

Sequencing reactions were performed with ABI PRISM dye terminator cycle sequencing reagents (Perkin-Elmer) and were analyzed on an ABI 377 machine. When an SSCP was identified, the amplified DNA was either sequenced directly or cloned (TA Cloning Kit; Invitrogen), and individual alleles were sequenced. Restriction-endonuclease digestions were performed according to the manufacturer's protocol.

Figure 3 *COL9A2* splice-site mutation in family K. *a,* Nucleotide sequence of exon 3 (*upper case*) /intron 3 (*lower case*) boundary. Shown at the top is the wild-type sequence; shown below is the sequence from the mutant allele. Indicated by an arrow is the G→A transition at the last nucleotide of exon 3. *b,* PAGE analysis of *COL9A2* exon3/intron3 genomic DNA fragment containing the mutation. The mutation disrupts a *Hph*I restriction-endonuclease site (indicated by an asterisk [*]). JK and his affected mother, UK, who are heterozygous for the mutation, show both digested and undigested product, whereas a control sample (lane C) shows only the digested product.

Figure 4 *COL9A2* splice-site mutation in family G. *a,* Nucleotide sequence of exon 3 (*upper case*) /intron 3 (*lower case*) boundary. Shown at the top is the wild-type sequence; shown below is the sequence from the mutant allele. Indicated by an arrow is the G→C transversion at position +5 of intron 3. *b,* PAGE analysis of *COL9A2* exon3/intron3 genomic fragment containing the mutation. The mutation creates a *Mae*III restriction-endonuclease site (indicated by an asterisk [*]). Affected individuals of family G, who are heterozygous for the mutation, show both undigested and digested product; a control sample (lane C) shows only the undigested product.

Results

Mutational Analysis in COL9A2

SSCP analysis of PCR-amplified genomic DNA containing the exon3/intron3 splice-donor site of *COL9A2* identified different aberrantly migrating bands in two unrelated individuals with MED (not shown). Direct sequence analysis identified novel point mutations in the exon3/intron 3 splice-donor site, which were confirmed by cloning and sequencing of individual alleles.

Patient JK (individual II-1 in family K) was heterozygous for a G→A transition at the last nucleotide of exon 3 (fig. 3*a*). This mutation disrupted a *Hph*I restriction-endonuclease site, and analysis of DNA from his affected mother confirmed that she was also heterozygous for this change (fig. 3*b*). Analysis of DNA from an affected member of each of the remaining 26 families with MED and from 20 unrelated control samples demonstrated the absence of this change, confirming that it was not a common polymorphism (data not shown).

Patient MG (individual III-1 in family G) was heterozygous for a G→C transversion at position +5 of intron 3 (fig. 4*a*). This mutation created a *Mae*III restriction-endonuclease site, and analysis of DNA from her affected sons (IV-1 and IV-2) confirmed that they were

also heterozygous for the change, whereas analysis of DNA from her unaffected husband confirmed that he did not have the mutation (fig. 4*b*). Analysis of DNA from an affected member of each of the remaining 26 families with MED and from 20 unrelated control samples demonstrated the absence of this change, confirming that it was not a common polymorphism (data not shown).

Analysis of mRNA Splicing

To study the effect of these mutations on the splicing of mRNA from the mutant allele, further blood samples were obtained from affected members of each family, for the establishment of Epstein-Barr virus (EBV)–transformed lymphoblastoid (LB) cell lines. *COL9A2* cDNA from exons 2–5 was amplified by PCR, and 6% PAGE analysis showed the presence of both a normal-sized DNA product and a shortened DNA product, suggestive of the loss of exon 3 (fig. 5). Individual clones from patients MG and JK were sequenced, which confirmed both the identity of the normal product and the loss of exon 3 sequence from the shortened PCR product.

In patient MG (Individual III-1 in family G), the normal and deleted PCR products were present in approximately equal amounts, which would be expected in a patient heterozygous for a splice donor–site mutation (fig. 5). However, in patients JK (fig. 5) and UK (not shown), the deleted PCR product was much less abundant than the normal-sized PCR product. To determine whether the reduction in the amount of the deleted PCR product was due to either normal splicing of the mutant mRNA or degradation of the misspliced mutant mRNA, PCR-amplified cDNA was digested with *Bpm*I. The recognition site for this restriction enzyme is at the exon 3/exon 4 boundary and would be predicted to cleave DNA, containing both exon 3 and the G→A transition at the last nucleotide (fig. 6*a*). Restriction-enzyme digestion failed to produce a DNA fragment of the expected size (~110 bp) or to reduce the amount of normal-sized DNA product (fig. 6*b*), which suggested that the normal-sized DNA product consisted of PCR-amplified cDNA derived entirely from the normal allele.

Mutation Screening in the COL9A1 *and* COL9A3 *Genes*

Because of the apparent grouping of EDM 2 mutations in the intron 3 splice-donor site of *COL9A2,* we decided to screen for mutations in the splice-donor sites of the equivalent exons of *COL9A1* and *COL9A3*. SSCP/heteroduplex analysis and *Hph*I restriction-endonuclease digestion of DNA from the exon 3/intron 3 splice-donor site of *COL9A3* failed to identify a mutation in any of the affected individuals whom we screened in the remaining 26 families with MED. The

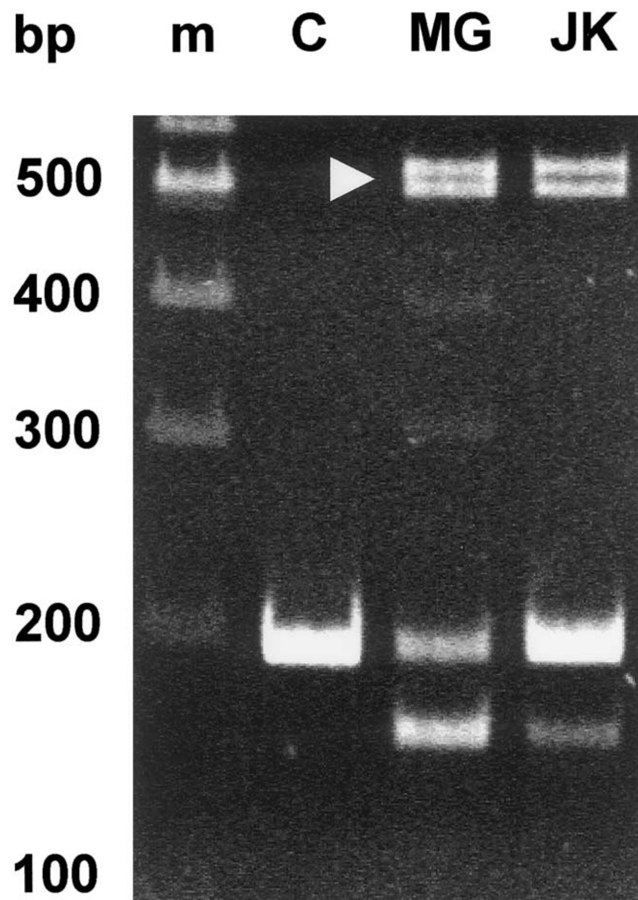

Figure 5 6% PAGE analysis of PCR-amplified *COL9A2* cDNA from a control sample (lane C) and from patients MG and JK. The control shows the expected DNA fragment of 196 bp, whereas both patient samples show both normal and shortened DNA fragments. The white arrow indicates putative heteroduplex molecules, formed between normal and deleted DNA fragments.

*Hph*I recognition sequence (*GGTGA*) covers 5 bp of the 7-bp intron 3 splice-donor–consensus sequence and, consequently, was a suitable restriction endonuclease for analysis of this site. SSCP/heteroduplex analysis of the exon 9/intron 9 splice-donor site of *COL9A1* identified the same aberrantly migrating bands in two unrelated individuals. DNA sequence analysis of the cloned PCR product identified a neutral polymorphism (T/C) at position +37 of intron 9 (data not shown), and both individuals were heterozygous for this polymorphic change.

Discussion

In this study we screened for mutations in specific regions of *COL9A1, COL9A2,* and *COL9A3* and identified two different point mutations in a splice-donor site of *COL9A2* that result in distinctive oligo-epiphyseal

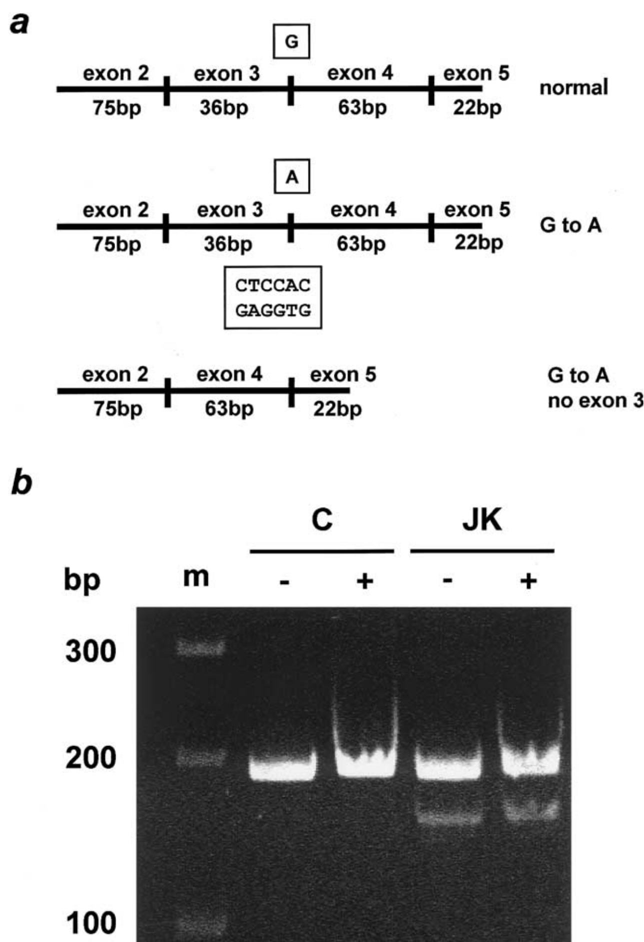

Figure 6 *Bpm*I restriction-enzyme digestion of *COL9A2* cDNA. *a,* Possible combinations of *COL9A2* mRNA transcripts. At the top is the normal transcript with exons 2–5 (partial) and G as the last nucleotide of exon 3 (boxed). In the middle is a normal-sized transcript (exons 2–5) but with A as the last nucleotide of exon 3 (boxed). Also enclosed in a box is the recognition sequence of *Bpm*I. At the bottom is the incorrectly spliced transcript showing the loss of exon 3. *b,* 6% PAGE analysis of *COL9A2* cDNA amplified by PCR from a control sample (lane C) and patient JK, before (−) and after (+) digestion with *Bpm*I.

forms of MED (MED type II). The mutations (Ex3 [−1] G→A and IVS3DS [+5] G→C) result in the skipping of exon 3 during pre-mRNA splicing and are predicted to lead to an in-frame deletion of 12 amino acid residues from the COL3 domain of the pro-α2(IX) chain. These data are in agreement with the only other *COL9A2* mutation reported to date (IVS3DS [+2] T→C), which also resulted in the skipping of exon 3 during pre-mRNA processing (Muragaki et al. 1996). Furthermore, mutations at positions −1 and +5 of the splice-donor sites of other procollagen genes have been described elsewhere (Human Gene Mutation Database) and have resulted in exon skipping and the synthesis of shortened pro-α chains. Mutations at the terminal nucleotide (−1) of exon 6 in the *COL1A1* and *COL1A2* genes have been identified in several cases of Ehlers-Danlos syndrome type VII (reviewed in Byers et al. 1997), and a similar G→A transition at the terminal nucleotide of exon 34 of *COL3A1* resulted in Ehlers-Danlos syndrome type IV (Kuivaniemi et al. 1995). In addition, mutations at the +5 position of splice-donor sites in the *COL1A1, COL1A2, COL2A1,* and *COL3A1* genes have been shown to result in osteogenesis imperfecta, spondyloepiphyseal dysplasia, and Ehlers-Danlos syndrome type IV, respectively (Bonadio et al. 1990; Ganguly et al. 1991; Lee et al. 1991; Bateman et al. 1994; Tiller et al. 1995; Nicholls et al. 1996). Interestingly, our data suggest that, in addition to skipping exon 3 during mRNA splicing, the Ex3 (−1) G→A mutation also results in the degradation of mRNA transcribed from the mutant allele. Our studies do not argue whether this degradation occurs during mRNA processing (because of the formation of unstable splicing intermediates) or after the completion of mRNA processing prior to or during protein translation. Degradation of mRNA from the mutant allele has been suggested as a possible disease mechanism in MED type II (van Mourik et al. 1998*b*), and, indeed, our data do support this hypothesis in affected members of family K. However, it is clear that this is not a suitable explanation for MED in either family G or the family reported by Muragaki et al. (1996). It seems likely that a number of mechanisms may contribute to the disease pathophysiology of MED type II, and this may ultimately be reflected in clinical variability even within this subgroup of MED.

The mutations presented in the present study confirm that abnormalities in α2(IX) can result in MED. Analysis of equivalent splice-donor sites in the *COL9A1* and *COL9A3* genes failed to identify mutations in affected individuals from the remaining 26 families with MED. Such findings would suggest that mutations in these regions of *COL9A1* and *COL9A3* are not a common cause of MED and that the contribution of these two genes to the molecular pathology of MED remains to be determined. It is likely that the identification of the causative mutation in most families with MED will require extensive screening of exons from the *COL9A1, COL9A2,* and *COL9A3* genes, particularly when cartilage mRNA is not available and linkage analysis does not implicate a specific candidate gene.

Affected members of families K and G have a distinctive oligo-epiphyseal form of MED, which is similar to the phenotype of affected individuals in the two families with MED type II that have been reported elsewhere (Barrie et al. 1958; Briggs et al. 1994; Muragaki et al. 1996; van Mourik et al. 1998*a*). In essence, affected individuals in all four families are characterized by normal or mild short stature and pain and/or stiffness of

the knees and hands. To some extent there is pain and/or stiffness of the ankles but not of the hips. In addition, varus or valgus deformities that develop at an early age in affected family members sometimes require surgical correction. These features are in direct contrast to the clinical and radiographic presentation of individuals with MED type I phenotypes resulting from mutations in the *COMP* gene (Oehlman et al. 1994; Briggs et al. 1995, 1998; Deere et al. 1995; Ballo et al. 1997; Susic et al. 1997; Loughlin et al. 1998). In the patients described in these reports, the major clinical symptom is severe hip dysplasia leading to premature osteoarthritis and joint replacement. Although individuals with MED type I suffer from knee pain and stiffness, and although radiographs show flattened and irregular distal femoral epiphyses, there appear to be none of the varus or valgus deformities that are associated with MED type II.

The classification of MED into the Fairbank or Ribbing types owes much to the individual interpretation of clinical and radiographic findings by clinical geneticists and/or radiologists. This may help to explain the variability that is seen in the diagnosis of MED phenotypes, and perhaps the identification of a genetic or molecular basis of disease may help in the future to confirm a particular diagnosis. In 1958, when Barrie et al. predicted that mutations in at least two genes were responsible for the phenotypic variability of MED, they suggested that additional series of x-rays would need to be studied to determine the extent of genetic heterogeneity in MED. The advent of molecular genetics has validated their original hypothesis, and we now suggest that abnormalities in COMP primarily produce severe forms of MED (MED type I), whereas certain mutations in the *COL9A2* gene produce milder forms of the disease (MED type II). It is interesting to note that the large family with relatively mild MED, originally reported by Barrie et al. (1958), was the first family with MED to be linked to *COL9A2* (Briggs et al. 1994).

The findings presented in the present study provide some rationale for the phenotypic variability seen in MED; however, the identification of additional mutations in both the COMP and type IX collagen genes will help to elucidate further the genetic and molecular basis of this heterogeneous disorder.

Acknowledgments

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Electronic-Database Information

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

Online Mendelian Inheritance in Man (OMIM): http://www.ncbi.nlm.nih.gov/Omim/ (for MED [MIM 226900], EDM 1 [MIM 132400], EDM 2 [MIM 600204], and EDM 3 [MIM 600969])

Human Gene Mutation Database http://www.uwcm.ac.uk/uwcm/mg (for mutations in human procollagen genes)

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