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Autosomal Dominant and Recessive Osteochondrodysplasias Associated with the *COL11A2* Locus

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Summary

Identifying mutations that cause specific osteochondrodysplasias will provide novel insights into the function of genes that are essential for skeletal morphogenesis. We report here that an autosomal dominant form of Stickler syndrome, characterized by mild spondyloepiphyseal dysplasia, osteoarthritis, and sensorineural hearing loss, but no eye involvement, is caused by a splice donor site mutation resulting in "in-frame" exon skipping within the *COL11A2* gene, encoding the α2(XI) chain of the quantitatively minor fibrillar collagen XI. We also show that an autosomal recessive disorder with similar, but more severe, characteristics is linked to the *COL11A2* locus and is caused by a glycine to arginine substitution in α2(XI) collagen. The results suggest that mutations in collagen XI genes are associated with a spectrum of abnormalities in human skeletal development and support the conclusion of others, based on studies of murine chondrodysplasia, that collagen XI is essential for skeletal morphogenesis.

Introduction

Molecular genetic analyses of osteochondrodysplasias, hereditary disorders affecting skeletal development in both mice and humans, promise to provide insights into the large number of genes essential for skeletal morphogenesis. With improved techniques for gene mapping, positional cloning, functional cloning, and mutation detection, the task of identifying the mutations causing these disorders is becoming less daunting. This is illustrated by the recent successes in identifying mutations in genes encoding structural, growth factor receptor, and sulfate transporter proteins as causes of distinct osteochondrodysplasias (Warman et al., 1993; Shiang et al., 1994; Reardon et al., 1994; Hästbacka et al., 1994). Adding to this is the accompanying paper by Li et al. (1995 [this issue of *Cell*]), which demonstrates that autosomal recessive chondrodysplasia (*cho*) in mice is due to a mutation in the gene, *Col11a1*, coding for one of the polypeptide subunits of the quantitatively minor fibrillar collagen XI in cartilage.

Collagen XI molecules are heterotrimers of three distinct subunits, α1(XI), α2(XI), and α3(XI), encoded by *Col11a1*, *Col11a2*, and *Col2a1*, respectively (Eyre and Wu, 1987). Analysis of *cho* mice demonstrates that the absence of α1(XI) collagen chains, and therefore collagen XI heterotrimers, leads to a severe disruption of the columnar arrangement and maturation of growth plate chondrocytes, abnormalities in collagen fibril diameter, and reduced cohesive strength of cartilage matrices (Li et al., 1995). These data suggest that the *Col11a1* gene is essential for skeletal morphogenesis.

Here, we report that the *COL11A2* locus, encoding the α2(XI) subunit of collagen XI, is associated with autosomal dominant and autosomal recessive human osteochondrodysplasias. We describe a mutation affecting a 5′ splice site leading to in-frame exon skipping and causing an autosomal dominant form of Stickler syndrome. We also show that an autosomal recessive disorder characterized by spondyloepiphyseal dysplasia and sensorineural hearing loss, similar to the otospondylomegaepiphyseal dysplasia (OSMED) syndrome, is linked to the *COL11A2* locus and is caused by a glycine to arginine substitution in α2(XI) collagen. These data suggest that mutations affecting collagen XI can cause a spectrum of clinical phenotypes and that collagen XI is essential also for human skeletal morphogenesis.

Results

Identification of the *COL11A2* Mutation in a Family with Stickler Syndrome

We recently described linkage to markers near the *COL11A2* locus in a large Dutch kindred with a Stickler syndrome phenotype (Brunner et al., 1994). All 16 affected

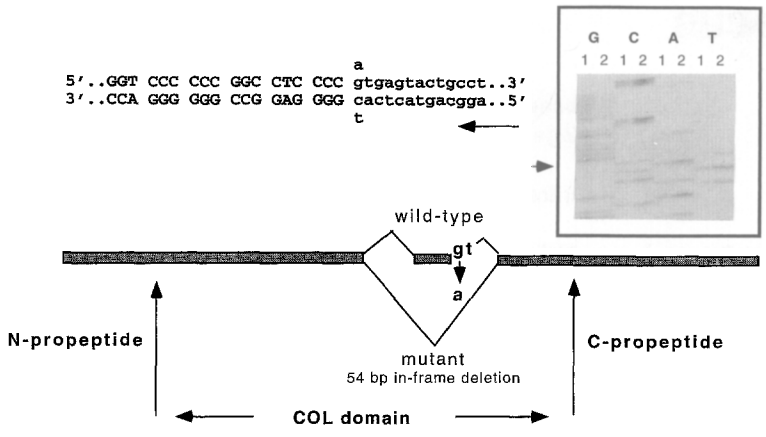

Figure 1. A Single Nucleotide Change in a Splice Donor Site in *COL11A2* in the Family with Dominant Osteochondrodysplasia

Schematic representation of the α2(XI) collagen mRNA showing the in-frame deletion of an exon caused by the G to A transition in the 5′ splice site downstream of the exon. This exon sequence is located 108 nt upstream of the junction between sequences encoding the triple-helical (COL) and C-propeptide domains of the α2(XI) chain. Genomic sequencing of the noncoding strand from a patient (lane 2) and a control (lane 1) is shown (top right). The arrowhead points to the mutation site. The genomic sequence covering the mutation site with exon sequences in capital letters and intron sequences in small letters are provided (top left). The arrow below the noncoding strand indicates the sequencing direction in the autoradiogram.

individuals in the family had characteristic facial features of Stickler syndrome (Stickler et al., 1965) combined with hearing impairment. Several patients had cleft palate and mild arthropathy, but none had the ophthalmological signs usually associated with the Stickler syndrome. To identify the mutation causing the disorder in this family, we used reverse transcription–polymerase chain reaction (RT–PCR) from total RNA extracted from chondrocytes and Epstein-Barr virus–transformed (EBV-transformed) lymphoblasts from patients heterozygous for the defective allele and from unaffected individuals. Primers for nested PCR were designed to cover, in five overlapping fragments, the whole coding sequence (4953 bp) of the α2(XI) collagen gene. Restriction enzymes were used to cut these ^{33}P-labeled amplification products into several smaller fragments to enhance the likelihood of detecting differences between the patient and the control samples. The digested fragments were analyzed on MDE (AT Biochem) and single-strand conformation polymorphism (SSCP) gels.

SSCP shifts and heteroduplexes were identified, and the corresponding fragments were sequenced. Several neutral polymorphisms and a double-sequence caused by deletion of 54 nucleotides were observed. Since the deletion corresponded to an exon in the triple-helical region of the α2(XI) gene, we tested the hypothesis that the deleted sequence represented a skipped exon during RNA splicing of α2(XI) transcripts. A novel primer pair was synthesized according to the sequences of exons adjacent to the skipped exon, and the corresponding region was amplified using patient genomic DNA as a template. Cycle-sequencing of this PCR product revealed heterozygosity for a 1 bp change at the exon–intron boundary such that the intronic donor-site sequence, GTGAG, was replaced by ATGAG (Figure 1). This change created a novel NlaIII site in the genomic sequence. To demonstrate that this sequence change was present in all the affected individuals, genomic DNAs of all family members were PCR amplified, the sense primer being end-labeled with ^{32}P. After NlaIII digestion, these products were run on 5% denaturing sequencing gels. The G to A transition cosegregated with the disease (Figure 2).

Linkage between an Autosomal Recessive Phenotype and Loci on Chromosome 6p

A second Dutch kindred has been identified, in which three affected siblings have severe degenerative joint disease (osteoarthritis), which presents in early adulthood and affects predominantly the hips, knees, elbows, and shoulders (Figure 3). The spine is less severely affected, and adult height is only slightly below that of the unaffected siblings. There is increased lumbar lordosis and prominent interphalangeal joints. Short fifth metacarpals are found in all cases. The patients have distinct facial features: midface hypoplasia with a short upturned nose, prominent eyes, depressed nasal bridge, and prominent supraorbital

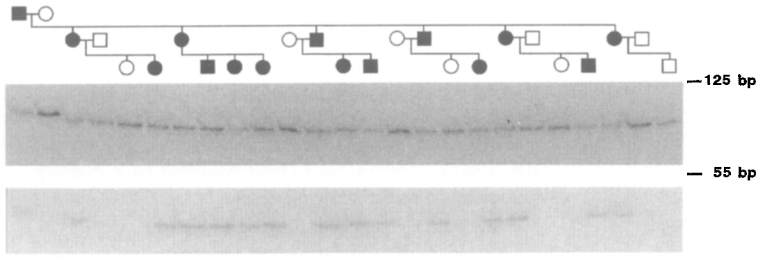

Figure 2. Cosegregation of the Phenotype and the Mutation in the Family with Dominant Osteochondrodysplasia

Cosegregation analysis of the splice site mutation in the Stickler family by NlaIII digestions of ^{32}P end-labeled PCR products. A novel restriction site, created by the mutation, is 55 bp downstream of the end-labeled primer. In wild-type DNA, an NlaIII site is located 125 bp downstream of the end-labeled primer. Closed symbols represent individuals with the disease phenotype.

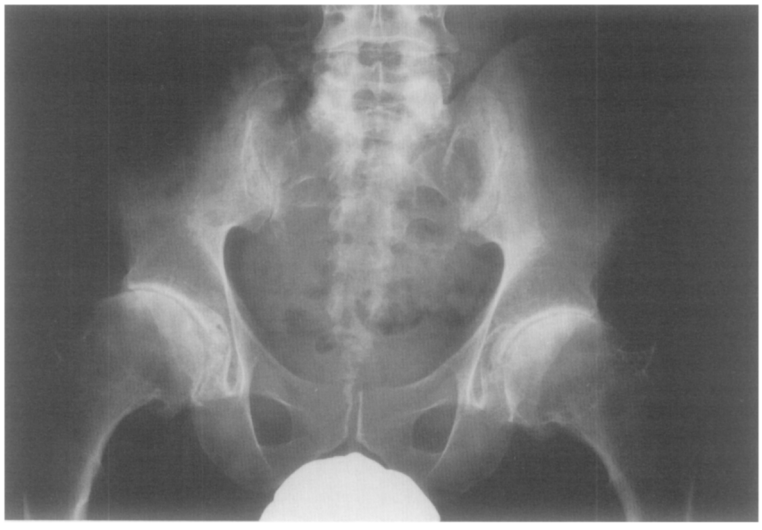

Figure 3. Severe Osteoarthritis at the Hip Joints of a Patient with Recessive Osteochondrodysplasia

Pelvic radiograph of patient 5 (aged 29 years) from the kindred with the autosomal recessive phenotype. Joint space narrowing, osteophyte formation, and osteosclerosis at the hips can be observed.

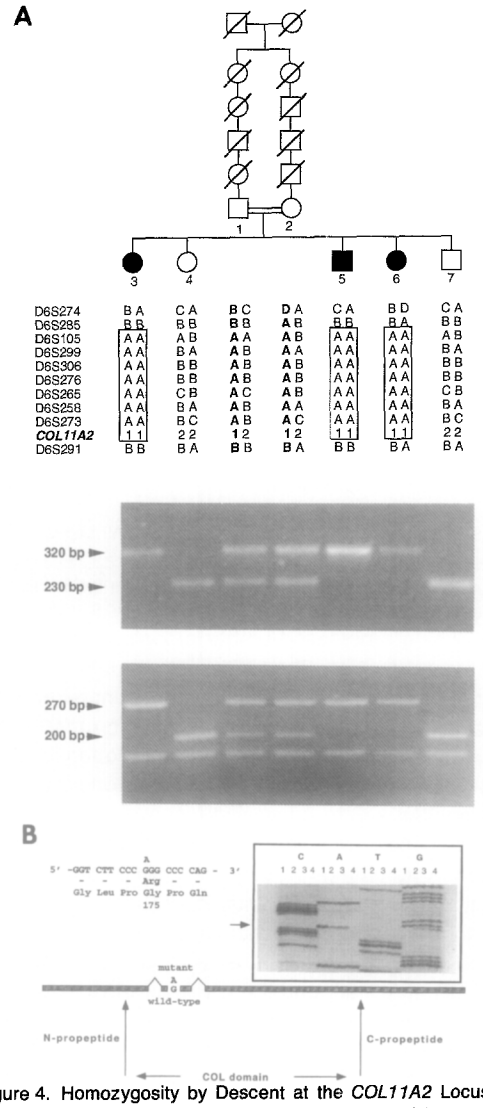

Figure 4. Homozygosity by Descent at the *COL11A2* Locus and a Single Nucleotide Change in the Coding Sequence of *COL11A2* in the Family with Recessive Osteochondrodysplasia

(A) Pedigree of the family with autosomal recessive osteochondrodysplasia (top). The genotype of each individual for 10 tested CA repeat markers from 6p21 is given. The haplotype associated with the disorder is in bold letters, and the haplotype for which the affected individuals are homozygous by descent is boxed. The result of the analysis of an intragenic MspI polymorphism (*COL11A2*) is shown in the photograph of an ethidium bromide–stained agarose gel (middle). MspI cleaves a 320 bp fragment into 230 bp and 90 bp (data not shown) fragments. The photograph of an ethidium bromide–stained agarose gel at the bottom of the figure shows the absence of an MspI site (note that this site is unrelated to the polymorphic site shown in the middle) in a genomic fragment due to the G to A transition in *COL11A2*. When digested with MspI, the 1.2 kb genomic PCR product is digested into fragments of 800 bp (data not shown), 270 bp, and 150 bp in affected individuals. In unaffected children, the 270 bp fragment is cleaved into fragments of 200 bp and 70 bp (data not shown). The parents show the presence of all fragments.

(B) Schematic representation of the α2(XI) collagen mRNA showing the G to A transition in the codon of Gly-175 as counted from the amino terminus of the triple helical (COL) domain of the polypeptide chain. The mutation is located within a 45 bp-long exon of COL11A2. Genomic sequencing of the coding strand from a patient (lane 1), parents (lanes 2 and 3), and an unaffected child (lane 4) is shown (top right). The arrow points to the mutation site. The exon sequence and deduced amino acid sequence covering the mutation site are provided (top left). The G to A transition changes the glycine codon to an arginine codon. Closed symbols represent individuals with the disease phenotype.

ridges. Sensorineural hearing loss is present from birth and requires the use of hearing aids in all three affected siblings. None of the patients have myopia or vitreoretinal degeneration. The pedigree of the family is shown in Figure 4A, and the clinical features are summarized in Table 1. Note that the parents of the affected siblings are fourth cousins.

The affected siblings were found to be homozygous for an extended haplotype of seven CA (dinucleotide) repeat polymorphisms from chromosome 6p21 near the *COL11A2* locus (Figure 4A). If the consanguinity loop was ignored, a maximum lod score of 1.45 without recombination was obtained for those markers that were fully informative (*D6S306*, *D6S276*, *D6S265*, *D6S273*). Higher lod scores

Table 1. Comparison of Clinical Features in the Families with Autosomal Dominant and Autosomal Recessive Phenotypes Linked to **COL11A2** with Those of the OSMED and Classical Stickler Syndrome

	Autosomal Recessive Kindred	OSMED Syndrome	Autosomal Dominant Kindred	Classical Stickler Syndrome
High myopia and vitreoretinal degeneration	Absent	Absent	Absent	Severe
Epiphyseal dysplasia and osteoarthritis	Severe	Severe	Mild	Mild
Vertebral involvement	Mild	Mild	Mild	Mild
Hearing loss	Moderate/severe	Moderate	Moderate	Absent/mild
Cleft palate (%)	Absent (0 of 3)	>50	25 (4 of 16)	<35
Midface hypoplasia and upturned nose	Yes	Yes	Yes	Yes
Inheritance pattern	autosomal recessive	autosomal recessive	autosomal dominant	autosomal dominant

were obtained, however, when the consanguinity loop was taken into account. Since for most of these markers allele frequencies are not available from the Dutch population and the frequency of the abnormal allele is also unknown, the lod score calculations were performed with a range of parametric values. Conservative estimates of 0.002 for the abnormal allele and 0.005 for the marker haplotype yielded a lod score of 3.09 at $\Theta = 0.0$.

Incorporation of *COL11A2* into the Human Linkage Map

Pairwise linkage analyses for *COL11A2* and loci on human chromosome 6 were performed to place the *COL11A2* gene into the human linkage map. As a marker for the *COL11A2* gene, we used an intragenic SSCP. Zero recombination was found between *COL11A2* and two loci, *D6S291* (Z = 3.01) and *D6S29* (Z = 7.65). The results of multipoint analysis are shown in Figure 5; the most favored order is [*HSPA1–D6S273*]–[*COL11A2–D6S29–D6S291–D6S439*]–*GLO1*. This order is consistent with the recent consensus map of chromosome 6 (Volz et al., 1994) and the second-generation Genethon map (Gyapay et al., 1994). It should be noted that the relationship between *D6S291* and *COL11A2* is based on only 10 meioses within three Centre d'Etudes du Polymorphisme Humain (CEPH) pedigrees informative for both loci.

In the family with recessive osteochondrodysplasia, patient number 6 has a recombination between *D6S273* and *D6S291* (see Figure 4A). Since we did not observe any recombinations between the *COL11A2* gene and the *D6S291* marker in the three informative CEPH families, we wanted to confirm that the gene is within the inherited haplotype. We have recently identified a novel polymorphism within the *COL11A2* gene that creates an MspI restriction site. A 180 bp genomic fragment containing this polymorphism was amplified from all family members. Both parents are heterozygous, and all children are homozygous for the two alleles identified, thus placing the observed recombination in patient number 6 centromeric to *COL11A2*, between the gene and the marker *D6S291* (see Figure 4A).

Identification of the *COL11A2* Mutation in the Family with Autosomal Recessive Osteochondrodysplasia

To find the mutation causing the autosomal recessive disorder, we used RT-PCR with total RNA extracted from

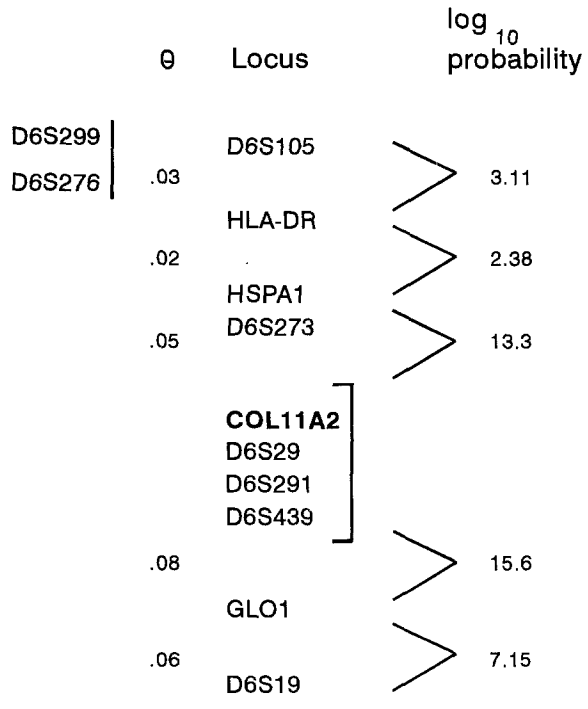

Figure 5. Partial Linkage Map around the *COL11A2* Locus

Multipoint linkage map of human chromosome 6p, incorporating *COL11A2*. The log 10 order probability for adjacent loci is indicated. Θ, sex average recombination frequency.

EBV-transformed lymphoblasts derived from parents and affected individuals. Several overlapping fragments were generated, and the complete coding sequence of the α2(XI) collagen gene was determined for one individual. This identified a G to A transition, converting a glycyl to an arginyl codon, within the triple-helical domain of α2(XI) collagen. This change in sequence eliminated an MspI restriction site within the genomic sequence. To demonstrate that this sequence change was present in all the affected individuals, genomic DNAs of all family members were PCR amplified, and the products were electrophoresed through a 4% agarose gel after digestion with MspI (see Figure 4A). The affected children showed the absence of the MspI site. A similar analysis with DNA from 63 unrelated parents (corresponding to 126 chromosomes) of CEPH families showed the presence of this MspI site in all samples (data not shown), thus excluding the possibility

that the G to A sequence change represented a common polymorphism. Finally, cycle sequencing of these genomic PCR products demonstrated that affected children are homozygous for the arginyl codon, while unaffected children are homozygous for the glycyl codon; both parents are heterozygous for the sequence change (see Figure 4B).

Discussion

We present evidence that the *COL11A2* gene is associated with both autosomal dominant and autosomal recessive human osteochondrodysplasias. These data suggest that collagen XI is essential for normal skeletal development, in agreement with the conclusions of Li et al. (1995) in their accompanying paper.

A Mutation in *COL11A2* Causes Autosomal Dominant Stickler Syndrome without Eye Involvement

We have identified a G to A transition at a splice donor site within the *COL11A2* gene, which cosegregates with an autosomal dominant Stickler syndrome phenotype in a large family. In vitro splicing experiments have previously demonstrated the importance of the five conserved nucleotides at the splice donor site for efficient and correct splicing of mRNA (Talerico and Berget, 1990), and similar mutations in α1(I) and α2(I) collagen genes have been implicated in other osteochondrodysplasias (reviewed by Kuivaniemi et al., 1991). The present mutation causes in-frame skipping of a 54 bp exon, encoding 18 amino acid residues within the triple helical domain of the α2(XI) collagen molecule (Figure 1). Approximately 50% of the *COL11A2* mRNA transcripts recoverable by RT–PCR from patient chondrocytes and EBV-transformed lymphoblasts contain the deleted exon (data not shown), suggesting that the mutation does not significantly affect mRNA stability. The mutated polypeptide chain may therefore be synthesized at a level comparable to that of the wild-type chain. Mutant α2(XI) chains would be 18 amino acid residues shorter than wild-type chains, but would contain the intact sequence of the carboxy-terminal propeptide domain. In fibrillar procollagens, including collagen XI, chain association during trimer assembly initiates at the carboxy-terminal propeptide domain (Dolz and Engel, 1990). Therefore, mutant α2(XI) chains are likely to associate with α1(XI) and α3(XI) chains during trimer assembly. Once associated, however, the 18 amino acid deletion within the triple-helical domain is likely to interfere with normal triple helix formation. Whether the phenotypic effect of this mutation is due to the rapid degradation of abnormally folded heterotrimers causing a deficiency of collagen XI or whether it is a consequence of copolymerization of abnormal molecules with normal collagen II, IX, and XI molecules in cartilage collagen fibrils requires further study.

Mutations affecting another collagen gene, *COL2A1*, have previously been identified in several families affected by Stickler syndrome having eye involvement, but genetic heterogeneity has been observed in other families (reviewed by Vikkula et al., 1994). Snead et al. (1994) have recently extended this observation by linking *COL2A1* in 20 additional families with eye involvement and excluding *COL2A1* in four families lacking congenital vitreous anomaly. Our results suggest that *COL11A2* is the likely candidate for these unlinked families.

Interestingly, although *COL2A1* mutations can result in a spectrum of osteochondrodysplasia phenotypes (reviewed by Vikkula et al., 1994), only haploinsufficiency mutations (i.e., premature stop codons) have been identified in the *COL2A1*-linked Stickler syndrome families. Since type II collagen accounts for 90% of total cartilage collagen, it is not surprising that haploinsufficiency could have a phenotypic consequence. Whether haploinsufficiency mutations affecting collagen XI, which comprises less than 10% of total cartilage collagen, have a similar effect cannot yet be determined.

A *COL11A2* Mutation Is Also the Cause of the Autosomal Recessive Phenotype

The three affected patients in the consanguineous family with autosomal recessive osteochondrodysplasia appear homozygous by descent for an interval defined by seven chromosome 6p21 CA repeat markers, within which we have mapped the *COL11A2* gene (Figure 4A). Using conservative estimates for mutant allele frequency (0.002) and extended haplotype frequency (0.005), a lod score of 3.09 with the *COL11A2* locus was obtained.

The phenotype of the affected members in this family, although more severe, resembles that of the family with the dominantly inherited *COL11A2* splice site mutation (Table 1). It also shares similarites with the OSMED syndrome (Giedion et al., 1982), which itself shares radiographic features with Stickler syndrome (Spranger, 1985). The clinical similarity among these disorders and the evidence of linkage to the *COL11A2* locus in our family strongly suggested that a mutation in *COL11A2* was responsible for the autosomal recessive phenotype as well.

The *COL11A2* mutation causing the autosomal recessive phenotype is likely to affect the stability of heterotrimeric collagen XI molecules since it changes a glycyl residue in a Gly–X–Y triplet to arginine. The location of the mutation within the triple-helical domain of α2(XI) collagen suggests that it creates, like the mutation in the autosomal dominant Stickler syndrome, a mutant polypeptide capable of participating in trimer assembly. This raises the question of why heterozygous "carrier" parents for the glycine to arginine mutation are asymptomatic, while the heterozygotes for the exon-skipping mutation show a clinical abnormality. We suggest that a possible reason for this difference is that substituting arginine for glycine close to the amino terminus of collagen XI molecules may still allow incorporation of mutated molecules into cartilage fibrils and therefore may allow some residual function, while a large in-frame deletion close to the carboxyl end of the molecule causes a complete disruption of triple-helical folding and function. Supporting evidence for this comes from mice heterozygous for the *cho* mutation (a functional null allele in *Col11a1*) that are asymptomatic, suggesting that a reduction in the level of functional collagen XI may not be clinically apparent. In contrast, however, homozy-

gosity for the *cho* mutation, leading to complete deficiency of *Col11a1*, has profound phenotypic consequences (Li et al., 1995).

In the mice, homozygosity for a functional null mutation in *Col11a1* results in a perinatal lethal chondrodysplasia. A partial loss of function would explain why the recessive mutation in *COL11A2* is not likewise lethal. In addition, there is a difference in utilization of α1(XI) and α2(XI) chains within type XI collagen heterotrimeric molecules. In mammalian vitreous, the *COL5A2* gene product, α2(V), replaces α2(XI), forming a collagen V/XI hybrid molecule (Mayne et al., 1993). This most likely accounts for the lack of eye involvement associated with *COL11A2* mutations and could lessen the effects of a *COL11A2* mutation if a similar substitution also occurs in extraocular tissues.

It is interesting to note that both parents in the family with the recessive syndrome did not show clinical abnormalities in articular cartilage, although there appears to be a history of osteoarthritis on the paternal side of the family. This obviously raises the possibility that mutations in collagen XI genes similar to the one described here may represent a predisposing factor in osteoarthritis.

Experimental Procedures

Analysis of the *COL11A2* Gene

RNA was extracted from EBV-transformed lymphoblasts or chondrocytes following the protocol of the REX total RNA extraction kit (U. S. Biochemical). One to two micrograms of total RNA was used as template for reverse transcription using the Superscript Preamplification System (GIBCO BRL). Oligo(dT)s or random hexamers were used as primers for the cDNA synthesis.

For analysis of the dominant syndrome, PCR primers were designed to amplify the approximately 4.95 kb cDNA in five overlapping fragments. Nested primers were used for second round PCR. Amplification for the first round was performed for 35 cycles, with one cycle consisting of 94°C for 30 s, 58°C for 30 s, and 72°C for 3 min, followed by a final extension step at 72°C for 10 min. Second round PCR reactions differed from first round reactions in that 40 cycles were used, primer annealing was at 64°C, and primer extension was for 2 min. The primers used were as follows (nucleotide positions counted from the major transcription start site are shown in parentheses): COL11A2-1 (sense), 5′-CGGTGGATGGAAGCCGTCTGA (nucleotides 4507–4527); COL11A2-2 (antisense), 5′-GTGACGTCATCCCTAGGCGT (nucleotides 4822–4803); COL11A2-6 (antisense), 5′-CGCCATGTCCTTTCTCTTCTC (nucleotides 5486–5466); COL11A2-7 (sense), 5′-GGGGGACCCATTGGTCGCCA (nucleotides 3039–3058); COL11A2-8 (antisense), 5′-TCTCCATCCTCTCCAGCCAC (nucleotides 3217–3198); COL11A2-9 (sense), 5′-GGCTCATAGTCTGCTCCCTG (nucleotides 128–147); COL11A2-10 (sense), 5′-GGCCCAGTTGGAGACCCTGG (nucleotides 1221–1240); COL11A2-11 (antisense), 5′-CCAGGGTCTCCAGTCGGTCC (nucleotides 2314–2295); COL11A2-12 (sense), 5′-CCCTCTGGACCTCAGGGACC (nucleotides 2082–2101); COL11A2-13 (antisense), 5′-GCGCCATTGGGTCCAGCTGG (nucleotides 3541–3522); DDI (sense), 5′-AAGGAGAGCCTGCAGTGTTG (nucleotides 1117–1136); and PO2 (antisense), 5′-GAATGGGAGCATGAGAGATGT (nucleotides 1325–1305).

The sets of primers used were as follows: 9/11, DDI/8, DDI/13, 6/7, and 1/6 (for this primer pair, the second round conditions were used) for the first round and 9/PO2, 10/11, 12/8, and 7/2 for the second round, respectively.

All PCR reactions were done in a total volume of 50 μl containing 1× PCR buffer, 200 μM dNTPs, 0.5 μM (each) primer, and 1 U of Taq polymerase. For SSCP and heteroduplex analyses, 0.5 μl of [α-^{33}P]dCTP (10 mM, 2000 Ci/mmol) was added to the reaction. All five fragments were cut into smaller fragments using a set of different restriction enzymes: (9/PO2: FokI, HinfI, NlaIII, StyI; 10/11: AvaI, FokI, HinfI, StyI; 12/8: ApaI, FokI, MspI, StyI; 7/2: ApaI, FokI, HinfI, StyI; and 1/6: AluI, FokI, HinfI, StyI). The digested products as well as the undigested full-length products were loaded both on MDE gels and on 5% nondenaturing polyacrylamide SSCP gels (Warman et al., 1993).

Fragments showing differences with either technique were further analyzed by dideoxy-nucleotide cycle sequencing (dsDNA Cycle Sequencing System, GIBCO-BRL) using ^{33}P end-labeled primer. Second-round PCR primers were used for the sequencing reactions. For amplifying genomic DNA, an additional primer set was synthesized: COL11A2-16 (antisense): 5′-TTGGCTCCTTTGGGGCCAGC (nucleotides 4381–4362); COL11A2-17 (sense): 5′-CCCTGGGCAGAAGGGTGAGA (nucleotides 4280–4299). Amplification conditions were 35 cycles at 94°C for 30 s, 58°C for 30 s, and 72°C for 1 min, with a final extension at 72°C for 10 min. The amplification product was also cycle sequenced.

For analysis of the recessive syndrome, several pairs of PCR primers were used to amplify the α2(XI) cDNA in seven overlapping fragments. In addition to primers that were used for analysis of the dominant syndrome (COL11A2-1, -6, -8, -9, -10, -11, -12, and -13), several other primers were also used, and they were as follows: COL11A2-14 (antisense), 5′-GTCGGTATGGCCTCATCTTCC (nucleotides 4552–4532); COL11A2-15 (sense), 5′-GATCCTGGCGCTATAGGTGCC (nucleotides 3981–4001); COL11A2-21 (antisense), 5′-CTCAGACCATCAGGGCCAGG (nucleotides 4066–4047); COL11A2-22 (sense), 5′-AGTGCAGGGTCCTGTGGGGC (nucleotides 3152–3171); COL11A2-23 (antisense), 5′-CCCTTCAGACCCCGAATTCCG (nucleotides 2161–2150); COL11A2-27 (sense), 5′-TCTGGGGAGAAGGGAGAAAC (nucleotides 3453–3472); and COL11A2-32 (antisense), 5′-CCTCGCCTGCTGCAGGATCG (nucleotides 1409–1390).

The first round of PCR was performed with the primer sets 9/11, 10/13, 13/22, 21/27, 14/15, and 1/6; for the second round, the PCR-nested primers were 9/32 (9/11-PCR used as a template), 12/8, and 23/10 (10/13-PCR used as a template). Amplification reactions and conditions were essentially the same as for the analysis of the dominant syndrome, except for minor adjustments of annealing temperature and extension times, as needed.

The PCR products were sequenced by cycle sequencing. Sequencing primers were as follows: COL11A2-2, COL11A2-6–COL11A2-17, -21, -22, -23, -27, -32, -36, and -37. Others used were as follows: COL11A2-5 (antisense), 5′-CCGAATGGACAGGATCAGAC (nucleotides 5204–5185); COL11A2-20 (sense), 5′-CCAACGGGTTTCCTGGACCG (nucleotides 2614–2633); COL11A2-26 (sense), 5′-CAGGGCATCCAGCGTCTCAG (nucleotides 161–181); COL11A2-28 (sense), 5′-AGGGAACCCTGGTCCTGTTG (nucleotides 3752–3771); COL11A2-29 (sense), 5′-TGCCTGGCTATCCTGGACGT (nucleotides 2362–2281); COL11A2-30 (sense), 5′-CACTCGCCAGCTTTTCCCAG (nucleotides 434–453); COL11A2-31 (sense), 5′-GGAGACTTAGGACCTCAGGG (nucleotides 1503–1522); COL11A2-33 (sense), 5′-AGAGCCAGGACCTCCTGGAC (nucleotides 1892–1911); COL11A2-34 (sense), 5′-GGTGATGTCCAGGAGCTGGC (nucleotides 828–847); and COL11A2-35 (antisense), 5′-TGATTCATAGGCTGCCTGGACC (nucleotides 881–860).

For amplification of genomic DNA from members of the family with the recessive syndrome, primers 23/33 were used to amplify a 1.2 kb product. This product was digested with MspI, and the digest was analyzed by electrophoresis through 4% agarose. The products were also sequenced by cycle sequencing using primers COL11A2-23 and COL11A2-38 (intronic sequence, sense): 5′-TCCGGGAGGCTGGATAGAAG.

Segregation of the Mutation with the Autosomal Dominant Phenotype

A primer, COL11A2-18 (sense): 5′-GGTATCCCAGGAGCATCCGG (nucleotides 4302–4321), was synthesized closer to the novel NlaIII site created by the G to A transition, and PCR was used to amplify a 180 bp fragment from genomic DNA covering the mutation site (primers COL11A2-16 and COL11A2-18). 35 cycles, each performed at 94°C for 30 s, 62°C for 30 s, and 72°C for 40 s, were done with a final extension step at 72°C for 10 min. The NlaIII site created by the mutation lies 55 bp downstream of the COL11A2-18 primer, whereas a NlaIII site in the wild-type sequence is 125 bp downstream. The COL11A2-18 primer was end labeled with ^{32}P, and genomic DNAs of all family members were amplified with the primer pair. The PCR prod-

uct was NlaIII digested and analyzed on a 5% denaturing polyacrylamide gel (Figure 2).

Linkage Analysis in the Family with Autosomal Recessive Osteochondrodysplasia

Genomic DNA was isolated from peripheral blood. To define the *COL11A2* locus in 6p21.3 (Kimura et al., 1989), we used CA repeat polymorphisms from 10 loci that are in the same or adjoining chromosome subband. PCR amplification of genomic DNA was used to analyze the marker loci using [α-^{32}P]dCTP in order to label the amplified DNA. Allelic bands were separated on a 6.6% denaturing polyacrylamide gel and visualized by overnight exposure of the dried gel to Kodak X-OMAT S film. The Mlink program package (version 5.03) was used for the calculation of lod scores (Lathrop et al., 1984) assuming autosomal recessive inheritance. In the calculations, the frequency of the disease allele and the frequencies of the marker alleles were varied as discussed in Results.

SSC and MspI Polymorphisms within the *COL11A2* Gene

The primer pair consisting of COL11A2-1 and COL11A2-2 was used to amplify an 898 bp genomic DNA fragment containing a 583 bp intron. Cycling conditions were 30 cycles of 95°C for 30 s, 58°C for 30 s, and 72°C for 1 min, with a 10 min 72°C final extension. SSC analysis of the full-length product identified a 3-allele polymorphism (with allele frequencies 0.76, 0.03, and 0.21, from largest to smallest allele size). This polymorphism was used to incorporate the *COL11A2* gene in the CEPH linkage map (Figure 5). Of 38 CEPH pedigrees, 12 were informative for the *COL11A2* SSC polymorphism. Pairwise and multipoint linkage analyses were performed between *COL11A2* and loci on human chromosome 6 (CEPH Database, version 7.0) using version 5.10 of the LINKAGE programs supplied by Dr. J. Ott (Lathrop et al., 1984). Allele frequencies were determined by genotyping 31 CEPH grandparents. Sex-specific recombination rates for males and females were set to be equal ($\Theta m = \Theta f$) for the pairwise linkage analysis. Two-point analysis was carried out using the CLODSCORE portion of the program, and locus order calculations were done using CILINK. Final order testing was done by testing the inverted orders using CILINK.

Another polymorphism, detected during SSCP and heteroduplex analysis, created a novel MspI restriction enzyme cutting site in the *COL11A2* cDNA. Two primers were synthesized to amplify the region containing this polymorphism from genomic DNA: COL11A2-36 (antisense), 5′-TGTAGGCCAATGGGTCCTGG (nucleotides 3436–3417) and COL11A2-37 (sense), 5′-TGTGGGGCAGCCTGGAGCAG (nucleotides 3308–3327). The PCR program used contained 30 cycles with 94°C for 30 s, 64°C for 30 s, and 72°C for 60 s, with a final extension at 72°C for 10 min. The length of the amplification product, when genomic DNA was used as template, was approximately 320 bp. The polymorphism creates a unique MspI site in this fragment, forming two restriction fragments, 230 bp and 90 bp long (Figure 4A).

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