Structural and segregation analysis of the type II collagen gene (*COL2A1*) in some heritable chondrodysplasias

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SUMMARY Seventy-seven persons with a variety of heritable chondrodysplasias were screened for gross rearrangements of the structural gene encoding the major cartilage collagen, collagen II. None was found. Segregation of the locus (*COL2A1*) was studied in 19 pedigrees using three restriction site dimorphisms (shown by *Pvu*II, *Hind*III, and *Bam*HI) and a length polymorphism as linkage markers. Discordant segregation between *COL2A1* and the mutant locus was seen in pedigrees with multiple epiphyseal dysplasia, autosomal recessive spondyloepiphyseal dysplasia tarda, hypochondroplasia, pseudoachondroplasia, diaphyseal aclasia, and trichorhinophalangeal syndrome. One pedigree with diastrophic dysplasia was weakly concordant. Autosomal dominant spondyloepiphyseal dysplasia tarda and metaphyseal chondrodysplasia (type Schmid) were not informative. We conclude that mutations of the collagen II gene are not a common feature of the heritable chondrodysplasias. Since the chondrocyte binding protein, chondrocalcin, is also encoded at *COL2A1* our conclusions apply equally to this gene.

The heritable chondrodysplasias are a very heterogeneous group of syndromes, the unifying features of which are abnormal development of the bones and joints, frequently associated with short stature.^{1,2} The criteria for delineating the recognised osteochondrodysplasias were agreed at the 1976 Paris conference on nomenclature.³ The degree of disability and handicap is very variable but it has been estimated that as many as 6000 persons are handicapped throughout life in the United Kingdom alone (population 5.5×10^7).⁴

The hypothesis that heritable skeletal dysplasias could sometimes be the result of abnormalities in extracellular matrix components has been supported by a combination of biochemical and, recently, genetic linkage evidence in osteogenesis imperfecta (OI).^{5,6} It has been shown that qualitatively different mutations at the two structural genes encoding collagen I, the major species in bone, can give rise to a wide range of OI phenotypes.^{7,8} We set out to investigate whether those heritable osteochondrodysplasias in which cartilage rather than bone was primarily involved could, in an analogous way, be

the result of mutations in the structural gene encoding collagen II which is the most abundant species in cartilage.

Collagen II (or type II collagen) is one of the three major fibrillar collagens found in man. All three exist in tissues as extracellular, stress resisting, cross linked polymers, the subunits of which are trimers of α chain polypeptides which form a tight hydrogen bonded triple helix. They perform a vital structural role in all tissues. Unlike the other two fibrillar collagens (collagens I and III) which are found in many different tissues, collagen II has a much more restricted distribution, being encountered only in hyaline and articular cartilage, the nucleus pulposus, and in the ocular vitreous. All three collagen II α chain polypeptides are identical and are encoded, in man, at a single locus, *COL2A1*, on the long arm of chromosome 12.

In the past decade, non-systematic histopathological and biochemical studies have suggested that in the chondrodysplasias the primary defect is in cartilage synthesis with consequent abnormalities of endochondral bone.⁹ A range of cartilage collagen abnormalities has been reported in the spondyloepiphyseal^{10,11} and spondyloepimeta-

physeal dysplasias,¹² diastrophic dysplasia,¹³ achondrogenesis and fibrochondrogenesis,¹⁰ and thanatophoric dwarfism.¹⁴ Not unexpectedly, altered proteoglycan metabolism has been reported in others, including pseudoachondroplasia, Kniest syndrome, spondylometaphyseal dysplasia (Kozlowski type), and atypical cases of spondylo-epiphyseal dysplasia.¹¹ However, the complex interrelationships between the various cartilage matrix components will always make identification of the primary defect difficult from biochemical studies alone.

To test the hypothesis that mutations at *COL2A1* cause heritable chondrodysplasias, we have used the cosmid clone cosHcolII which contains the entire 30 kb collagen II gene and 7 kb of flanking sequence.¹⁵ In common with the other major fibrillar collagen genes, the 5 kb of coding sequence is distributed between 50 to 51 exons. We have used this cosmid and subclones derived from it to search for gross rearrangements of *COL2A1* in multiple overlapping digests of DNA from chondrodysplasia (CD) patients. In addition, we have used restriction

site and length polymorphisms at this locus as genetic markers in segregation analysis of CD pedigrees. Using this method, we have already reported independent segregation of *COL2A1* and achondroplasia.¹⁶

Patients

Seventy-seven subjects with a variety of chondrodysplasias were identified from four skeletal dysplasia research clinics. All subjects were examined by at least one of the authors and relevant radiographs were taken, often serially. Nineteen potentially informative pedigrees were investigated. Details of the patients and kindreds studied are shown in tables 1 and 2. Pedigrees 1-1 (multiple epiphyseal dysplasia), 6-1 (pseudoachondroplasia), and 7-1-1 (trichorhinophalangeal syndrome) have featured in previous clinical reports.¹⁷⁻²⁰

Methods

DNA was prepared from frozen peripheral blood,

TABLE 1 Patients studied including their clinical and radiological features.

Disorder	Clinical features	Radiological features
Multiple epiphyseal dysplasia	Normal or mild short stature. Premature osteoarthritis. Mild joint deformities	Small, irregular, delayed epiphyses
Spondyloepiphyseal dysplasia tarda	Mild, short trunk dwarfism. Premature osteoarthritis particularly of the hips	Platyspondyly. Severe epiphyseal changes in the large proximal joints
Spondyloepiphyseal dysplasia congenita (mild coxa vara)	Marked short trunk dwarfism (120-140 cm). Osteoarthritis of the hips	Platyspondyly. Coxa vara with severe epiphyseal changes in large proximal joints
Atypical spondyloepiphyseal dysplasia	Abnormal from 2 years. Scoliosis. Severe osteoarthritis of hips. Pronounced myopia	Platyspondyly. Epiphyseal changes in hips and knees
Spondylometaphyseal dysplasia	Pectus carinatum, scoliosis. Moderate joint laxity. Severe shoulder and hip involvement	Platyspondyly. Metaphyseal splaying
Pseudoachondroplasia	Severe short limbed dwarfism from 2 years. Marked joint laxity. Severe premature arthritis of large joints	Fragmentation of epiphyses. Mushroom shaped metaphyses. Anterior vertebral beaking. Platyspondyly
Trichorhinophalangeal syndrome	Mild short stature. Minor deformity of interphalangeal joints. Pear shaped nose. Poor growth of hair	Brachydactyly. Cone shaped epiphyses. Widened metaphyses in femoral necks
Metaphyseal chondrodysplasia (type Schmid)	Mild lower limb shortness. Bowed femoral necks requiring osteotomy	Metaphyseal splaying and cupping. Coxa vara. Normal epiphyses
Hypochondroplasia	Mild short stature. Normal face. Spinal stenosis in one case	Flaring of metaphyses. Mild lumbar interpedicular narrowing
Diaphyseal aclasis	Normal stature. Palpable bony outgrowths around joints	Overgrowth of bone adjacent to the growth plate
Diastrophic dysplasia	Severe short stature. Joint contractures. Talipes equinovarus. Hitchhiker thumbs. Cauliflower ears	Scoliosis. Small rib cage. Symphalangism. Delayed epiphyseal ossification. Broad metaphyses
Dyschondrosteosis	Mesomelic shortening. Madelung-like deformity. Mild short stature	Madelung-like deformity
Chondrodysplasia calcificans punctata (Conradi syndrome)	Short limbed, short stature. Flat face. Mental retardation	Punctate stippling of epiphyses. Metaphyseal cupping and splaying
Kniest syndrome	Pronounced short stature. Joint contractures. Flat face. Myopia	Much enlarged metaphyses. Flattened vertebrae. Delayed ossification of femoral heads
Chondroectodermal dysplasia (Ellis-van Creveld syndrome)	Severe short stature. Polydactyly. Congenital heart defects	Short ribs and long bones. Coned epiphyses
Frontometaphyseal dysplasia	Bowed long bones. Enlarged supraorbital ridges	Frontal skull, bone overgrowth. Failure of metaphyseal modelling. Platyspondyly

TABLE 2 Summary of patients and kindreds studied and the results of segregation analysis expressed as lods at zero recombination distance. Where the mode of transmission was not clear from the pedigrees (2·1, 3·1, 3·2, 5·1, 5·2, 11·1) lods were calculated for both autosomal dominant (AD) and autosomal recessive (AR) inheritance. Results from achondroplasia have already been published¹⁶ and are included for completeness.

Disorder	Cases	Kindreds	Inheritance	Lods at $\theta=0.00$		
				1	2	3
Multiple epiphyseal dysplasia	25	2	AD	—∞	—∞	
Spondyloepiphyseal dysplasia (tarda)	6	3	AR	—∞	—∞	
			AD	—∞	NI	NI
Spondyloepiphyseal dysplasia (congenita)	4	2	AR	—∞	—∞	
			AD	NI	NI	
Atypical spondyloepiphyseal dysplasia	2	1	AR	0.12	—∞	
Spondylometaphyseal dysplasia	2	2	AR	—∞	—∞	
			AD	NI	NI	
Pseudoachondroplasia	6	3	AR	0.2	0.12	
Trichorhinophalangeal syndrome	6	1	AR	—∞	—∞	NI
Metaphyseal chondrodysplasia (Schmid)	6	2	AD	—∞	—∞	
Hypochondroplasia	4	1	AD	NI	NI	
Diaphyseal aclasis	6	1	AD	—∞	—∞	
Diastrophic dysplasia	2	1	AD	—∞	—∞	
Dyschondrosteosis	3		AR	0.24		
Chondrodysplasia calcificans punctata (Conradi syndrome)	2					
Kniest syndrome	1					
Chondroectodermal dysplasia (Ellis-van Creveld syndrome)	1					
Frontometaphyseal dysplasia	1					
Achondroplasia	17	3	AD	—∞	—∞	NI

then restricted, separated by electrophoresis, and blotted onto nitrocellulose filters using our usual modifications of standard conditions.²¹ Hybridisation probes were labelled with ³²P by either nick translation or random primer directed synthesis. Hybridisations were carried out overnight at 42°C in a mixture containing 50% v/v formamide, 5% w/v dextran sulphate, and 200 µg ml⁻¹ heparin after prehybridisation of the filters with the same mixture without dextran sulphate. When the whole cosmid was used as a probe, hybridisation to highly repeti-

tive sequences was reduced by including 20 µg ml⁻¹ denatured human DNA in the hybridisation mix.

GROSS REARRANGEMENT OF COL2A1

We used a combination of digestion with *Eco*RI, *Bam*HI, and *Pvu*II to screen for major rearrangements in *COL2A1*. A partial restriction map of the locus is illustrated in fig 1. Whereas we agree with the revision of the original *Eco*RI map²² by Sangiorgi *et al*²³ we disagree on the position of *Bam*HI sites, both with the original map (substan-

FIG 1 Partial restriction map of *COL2A1*. The limits of the collagen II gene are shown by arrows. Starred *Bam*HI, *Hind*III, and *Pvu*II sites are dimorphic. The solid box denotes the position and limits of the length variable segment. ND on the *Kpn*I and *Pvu*II maps refers to unmapped regions of the locus.

tially) and with the revision of Sangiorgi *et al*²³ in the ordering of the 6.5 kb and 6.3 kb fragments. Using *EcoRI* and *BamHI* digestion, the gene is divided into fragments of not more than 5.3 kb. In this laboratory we expect to detect an insertion or deletion of 200 bp in a 5.3 kb fragment and set this as the lower size limit of our screen. *PvuII* divides the gene into at least 14 fragments but the map of *PvuII* sites is not yet complete. Therefore, although fragmentation with this enzyme improves the sensitivity of the screen in certain regions, we cannot be sure that it does so throughout the gene.

SEGREGATION ANALYSIS

Segregation of *COL2A1* alleles was analysed in the pedigrees using three restriction site dimorphisms within the gene and a length polymorphism just beyond its 3' end. We have previously described the restriction site dimorphisms shown by *HindIII* and *PvuII*.^{24 25} Here we describe one further dimorphism shown by *BamHI*. Fig 2a shows the fragmentation pattern in the three different genotypes. The variable *BamHI* site has been mapped to a position 17 kb upstream of the 3' end of the gene (fig 1). The most recent allele frequency estimates for all three dimorphisms in unrelated English Caucasians are shown in table 3.

The length polymorphism was first noticed as an apparent deletion in one allele in four patients with lethal osteogenesis imperfecta.²⁶ Studies in this laboratory showed that the 'deletions' were the result of variation in the length of a DNA segment 1.0 to 1.6 kb downstream from the termination codon.²¹ This segment is composed of tandemly repeated oligonucleotides of core lengths of 31 and 34 bp.²⁷ Although short alleles are much more frequent in some non-European populations, length variation can be detected in English whites and we have used this variation, shown by *PvuII* digestion and hybridisation to E7 (fig 2b), the 3' most *EcoRI* fragment of cosHco1I, as a further genetic marker for *COL2A1*. The map positions of all four marker systems are shown in fig 1.

Results

No rearrangements of *COL2A1* were detected in any patient. Segregation of *COL2A1* using the linkage markers described above is shown in fig 3 and summarised in table 2. Discordant segregation was seen in multiple epiphyseal dysplasia (autosomal dominant, 1.1 and autosomal recessive, 1.2 types), spondyloepiphyseal dysplasia tarda (autosomal recessive, 2.1, 4.1), pseudoachondroplasia (autosomal recessive, 6.1), trichorhinopharyngeal syndrome (7.1), hypochondroplasia (9.1),

FIG 2 (a) Fragments generated by the *BamHI* site dimorphism. The three genotypes are shown. Fragment sizes are in kilobases. (b) Fragments generated by the 3' length polymorphism detected by hybridisation with fragment E7 after *PvuII* digestion. Tracks 1 and 3 show unresolved 'homozygotes' for alleles of length 2.5 and 2.4 kb respectively. Tracks 2 and 4 show resolved heterozygotes.

Structural and segregation analysis of the type II collagen gene

FIG 3 Pedigrees and COL2A1 genotypes. Letters at the top left of each pedigree denote the marker system used. H=HindIII, B=BamHI, P=PvuII, L=length polymorphism. Details of fragment sizes and allele frequencies are shown in table 3.

TABLE 3 Markers at *COL2A1*. Fragment lengths in kilobases generated by the three restriction site dimorphisms (H, P, B) and the allele frequencies estimated from unrelated Caucasian chromosomes. The variants generated by the length polymorphism (L) were shown by *PvuII* digestion. The apparently most frequent allele, designated 1, generates a 2.5 kb fragment, while other alleles, designated 2 in any pedigree, generate shorter fragments. The number of distinguishable alleles in the population and their frequencies have not been estimated in this study.

Notation	Enzyme	Allele 1		Allele 2		SE	Unrelated chromosomes studied
		Fragment(s)	Frequency	Fragment(s)	Frequency		
H	<i>HindIII</i>	7.0+7.0	0.47	14.0	0.53	0.03	316
P	<i>PvuII</i>	3.3	0.48	1.6+1.7	0.52	0.03	334
B	<i>BamHI</i>	6.3+1.1	0.07	7.4	0.93	0.02	148
L	<i>PvuII</i>	2.4		<2.4			

and diaphyseal aclasis (10.1). In one pedigree with pseudoachondroplasia (6.3), exclusion relied on the disease being inherited as an autosomal recessive from the common ancestor of a consanguineous marriage, because the patient had inherited different *COL2A1* alleles from his parents. Discordant segregation in one of the spondyloepiphyseal dysplasia congenita pedigrees (3.2) depended on the relatively unlikely event that the disorder was inherited as an autosomal recessive trait. The kindred was of insufficient size to ascertain the mode of inheritance independently.

Two families with metaphyseal chondrodysplasia (type Schmid) (8.1, 8.2) and two with spondyloepiphyseal dysplasia tarda (autosomal dominant, 2.2, 2.3) were uninformative because key persons were homozygous for all markers.

In four kindreds, spondyloepiphyseal dysplasia (congenita) (3.1), spondylometaphyseal dysplasia (5.1 and 5.2), and diastrophic dysplasia (11.1), concordant segregation was observed with weakly positive lod scores. However, these results only held if recessive inheritance were operating in these kindreds.

Discussion

We have used a crude estimate of *COL2A1* structural integrity so that the absence of observable gross rearrangements of the gene in CD patients should not be taken to mean that the gene does not contain the causal mutation. Gross rearrangements of collagen genes seem to be uncommon and only very rarely would a similar screen have detected mutations at the two collagen I loci in osteogenesis imperfecta, even though both fine structure and linkage data identify these genes as the mutant loci in this disorder.

Discordant segregation in well defined pedigrees is a more secure demonstration that *COL2A1* is not the mutant locus. In the absence of special factors, for which no evidence exists, the expected recom-

bination fraction between any of the linkage markers used in the analysis and the physical limits of the 30 kb gene is about 0.0003, so that even a single recombination event between these markers and the CD gene in a pedigree effectively excludes *COL2A1* as the mutant locus. Recently, it has been shown that chondrocalcin, a protein promoting the binding of chondrocytes to cartilage matrix, is identical to the C-propeptide of collagen II, which is cleaved from the mature collagen molecule during post-translational processing.²⁸ Since this peptide is also encoded at *COL2A1* and is entirely contained within cosHcoll, our structural and linkage results on the collagen II gene equally apply to the chondrocalcin gene.

In certain families, it was not possible to determine the inheritance pattern and in these cases we analysed the data separately for dominant and recessive transmission rather than relying on published mechanisms. Pseudoachondroplasia, for instance, can be inherited as an autosomal dominant or recessive trait with incomplete distinction between the two mechanisms on clinical grounds alone.²⁰

With these reservations we have excluded *COL2A1* as the mutant locus in several CD families. The evidence is now very strong against *COL2A1* as the mutant locus in achondroplasia¹⁶ and, therefore, our result in hypochondroplasia was not surprising since the two are thought to be allelic.²⁹ The striking abnormalities of cartilage development leading to premature osteoarthritis and deformity make multiple epiphyseal dysplasia a candidate for defects in *COL2A1* but our results in two families, one with autosomal dominant and one with recessive inheritance, did not support this. Bearing in mind the presence of collagen II in the vitreous, the ocular involvement of spondyloepiphyseal dysplasia suggests that *COL2A1* might be the mutant locus. The pedigrees here were not particularly informative but discordance was convincingly shown in an atypical kindred (4.1) where myopia was pronounced.

Further work is needed to explore the possibility that mutations in *COL2A1* are causal in the rarer dysplasias including those that we have studied. Obviously larger kindreds and further genetic markers would be valuable. It was particularly disappointing, for instance, that two potentially useful kindreds with spondyloepiphyseal dysplasia tarda (2.2, 2.3) and two with metaphyseal chondrodysplasia (type Schmid) (8.1, 8.2) were not informative because the alleles could not be distinguished in key persons. Diastrophic dysplasia merits further attention, not least because of the collagen changes which have previously been described.¹³ The small pedigree we studied did not exclude linkage to *COL2A1* but many more cases would need to be examined to achieve a significant positive lod score. There is also doubt about the method of inheritance of this dysplasia which is said to be recessive,²⁹ although we know of no cases of recurrence in sibs from the UK. Perhaps only families with intrinsic evidence of recessive inheritance should be studied, a daunting task in such a rare disease.

In summary, although *COL2A1* is a very plausible candidate locus in the heritable chondrodysplasias, our results find no evidence to support this and, in several cases, strong evidence against it. Certainly, the hypothesis that the different CD phenotypes might generally be the result of different mutations in the collagen II gene is now refuted.

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