Mutations within the gene encoding the $\alpha 1$(X) chain of type X collagen (COL10A1) cause metaphyseal chondrodysplasia type Schmid but not several other forms of metaphyseal chondrodysplasia

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Abstract
Type X collagen is a homotrimer of $\alpha 1$(X) chains encoded by the COL10A1 gene. It is synthesised specifically and transiently by hypertrophic chondrocytes at sites of endochondral ossification. Point mutations and deletions in the region of the COL10A1 gene encoding the $\alpha 1$(X) carboxy-terminal (NC1) domain have previously been identified in subjects with metaphyseal chondrodysplasia type Schmid (MCDS). To determine whether mutations in other regions of the gene caused MCDS or comparable phenotypes, we used PCR followed by SSCP to analyse the coding and promoter regions of the COL10A1 gene, as well as the intron/exon boundaries of five further subjects with MCDS, one subject with atypical MCDS, and nine subjects with other forms of metaphyseal chondrodysplasia. Using this approach, three of the subjects with MCDS were found to be heterozygous for the deletions 1864delACTT, 1956delT, and 2029delAC in the region of COL10A1 encoding the NC1 domain. These deletions would lead to alterations in the reading frame, premature stop codons, and the translation of truncated protein products. A fourth subject with MCDS was found to be heterozygous for a single base pair transition, T1894C, that would lead to the substitution of the amino acid residue serine at position 600 by proline within the NC1 domain. We did not, however, detect mutations in the coding and non-coding regions of COL10A1 in one subject with MCDS, the subject with atypical MCDS, and in the nine subjects with other forms of metaphyseal chondrodysplasia. We propose that the nature and distribution of mutations within the NC1 domain of COL10A1 causing MCDS argues against the hypothesis that the phenotype arises simply through haploinsufficiency but that an, as yet, unexplained mutation mechanism underlies this phenotype.

Key words: mutation; chondrodysplasia; collagen.

Type X collagen is a short chain, non-fibrillar collagen that consists of three identical $\alpha$ chains. The human $\alpha 1$(X) chains are encoded by a single gene, COL10A1, which has been cloned, sequenced, and localised to chromosome 6q21-q22.3,5 The homotrimer contains a short triple helical domain flanked by a large, globular carboxy-terminal (NC1) domain and a short amino-terminal (NC2) domain (fig 1). The formation of the type X collagen molecule, as with other collagen types, is thought to initiate at the NC1 domain, the structure of which ensures the correct association and alignment of the $\alpha$ chains before triple helix formation, which proceeds in an NC1 to NC2 direction.6 4 The NC1 and NC2 domains of the type X collagen molecules are retained extracellularly and participate in interactions between molecules to form supramolecular, hexagonal, lattice structures.9

During normal development, type X collagen is exclusively synthesised by hypertrophic chondrocytes of the growth plate during the process of endochondral ossification and its synthesis ceases with the fusion of the growth plates during puberty.10 11 Type collagen synthesis is, however, observed in the adult as a consequence of the reintiation of the endochondral ossification process in disease processes such as osteoarthritis and fracture repair.12 13 The precise function of type X collagen is not known but its specific expression within the growth plate has led to the assumption that it has a role in endochondral bone formation.

Genetic approaches to investigate the role of type X collagen in normal development and disease processes have included the generation of type X collagen null mice,14 the generation of mice transgenic for a chick type X gene with an in frame deletion of the region encoding part of the triple helical domain,15 as well as the search for mutations of COL10A1 that cause forms of human chondrodysplasia.16-22 The type X collagen null mice and the transgenic mice expressing the type X mini-gene were found to have different phenotypes. The type X collagen null mice were indistinguishable from control mice in terms of fertility, viability, long bone growth and development, as well as histology of the growth plate.14 In contrast, the mini-gene transgenic mice had a form of spondylometaphyseal dysplasia associated with haematological abnormalities.15

Mutations causing a human form of chon-
Mutations within the gene encoding the α1(X) chain of type X collagen (COL10A1)

Figure 1  Diagram of the type X collagen protein (above) indicating the collagenous and non-collagenous (NC) domains and the encoding gene, COL10A1 (below). The boxed area within the NC1 domain of the protein indicates the restricted region of the protein that is altered by mutations that have been found within the corresponding region of COL10A1 in subjects with MCDS. The boxed regions of the gene represent the exons, the lines the introns, and the hatched areas indicate regions of the gene that are not drawn to scale.

drodysplasia, metaphyseal chondrodysplasia type Schmid (MCDS), have been identified in several subjects and, in all reported incidences, these mutations occur exclusively in the region of the gene encoding the NC1 domain.\(^{16-22}\)

MCDS is a well characterised disorder of autosomal dominant inheritance which usually presents in early childhood. Affected subjects are of short stature and have genu varum and coxa vara.\(^{23,24}\) The mutations within the NC1 encoding domain that have been found to cause MCDS have included single base pair changes leading to the substitution of amino acids and deletions that alter the reading frame of the gene and introduce premature stop codons. The nature and location of the mutations causing MCDS, as well as recent in vitro data showing that abnormal type X collagen chains are not incorporated into collagen trimers, have led to the hypothesis that the MCDS phenotype results from haplosufficiency.\(^{16,18,21}\) To determine whether mutations in regions of the gene other than that encoding the NC1 domain caused MCDS (as would be expected if haplosufficiency were responsible for the phenotype) and whether similar conditions might be caused by other types of mutations in COL10A1 (as might be expected from the phenotype of the transgenic mice), we used PCR followed by SSCP to analyse the coding, intron/exon boundaries, and promoter regions of the COL10A1 gene of five further subjects with MCDS, one with atypical MCDS, and nine with other forms of metaphyseal chondrodysplasia. Mutations within the NC1 domain were detected in four of the five subjects with MCDS but not in the coding and non-coding regions of COL10A1 of the subject with atypical MCDS nor of the nine subjects with other forms of metaphyseal chondrodysplasia. We argue that the exclusive occurrence of mutations within the NC1 domain of COL10A1 causing MCDS, as well as the reported phenotypes of the type X null and transgenic mice, argue against the hypothesis that the MCDS phenotype arises simply through haplosufficiency.

Patients and methods

Patients

The pedigrees of the families investigated are given in fig 2. The affected subjects from families A to E had a phenotype that was entirely consistent with that of metaphyseal chondrodysplasia type Schmid.\(^{23,24}\) The phenotype of family B has been previously described.\(^{25}\) The affected subjects from families F to O had phenotypes that can be assigned to the broad category of the metaphyseal and spondylometaphyseal chondrodysplasias.\(^{26,27}\) Because of the heterogeneity of these conditions and the difficulty of making precise diagnoses, brief clinical descriptions of the affected subjects and their families are given in addition to the clinical category to which they can best be assigned.

Family F

The proband presented at 1 year of age with bilateral coxa vara which required osteotomies for correction during childhood. At the age of 12 years, she was noted to have a hemivertebra (ninth thoracic) with consequent scoliosis. She had marked short stature (an adult height of 120 cm) with nearly equal upper and lower segments. The proband's younger son was diagnosed at birth with infantile coxa vara. At the age of 6 years his radiographs showed metaphyseal chondrodysplasia of the knees. He had marked short stature and at the age of 19 years was 120 cm tall. The proband's older son and husband were unaffected. The condition in this family appeared to represent a form of metaphyseal chondrodysplasia of autosomal dominant inheritance. The condition resembled MCDS but was atypical in that the proband had a congenital vertebral anomaly and both she and her son were of shorter stature than is usual in classical MCDS. The diagnosis was metaphyseal chondrodysplasia type Schmid, atypical.

Family G

The proband's phenotype has been previously reported by David et al.\(^{28}\) In brief, the proband presented at birth with distinctive facies, skin pigmentation, hirsutism, and severe scoliosis. She was short in stature, had valgus knees and ankles and enlarged wrists, elbows, knees, and ankles. In addition she was epileptic. Her x rays were similar to metaphyseal chondrodysplasia type Jansen in that the metaphyses were enormously expanded. She was born to non-con- sanguineous parents and her younger sister was normal. Inheritance could be autosomal recessive or a new dominant mutation. The diagnosis was Patterson syndrome.

Family H

The proband presented at the age of 2 years with an abnormal gait. Radiology confirmed metaphyseal changes at the hips with coxa vara.
but these changes were minimally present in other regions of the skeleton. She developed idiopathic type scoliosis with double primary curves (low right thoracic and low left lumbar). In addition she had a small ventriculoseptal defect. She was born to normal non-consanguineous parents and had a younger unaffected sister. Inheritance of the condition was not known. The diagnosis was mild metaphyseal chondrodysplasia.

Family I
The proband presented at the age of 9\(\frac{1}{2}\) years with pain in the left hip and reduced movement. His MZ twin brother did not have any symptoms. Radiographs of both boys showed metaphyseal changes at the hips and knees and slight reduction of vertebral height posteriorly. The proband had a superimposed Perthes disease on the left side. Both boys had pectus excavatum. Their heights at that time were on the 25th centile. Their heights at the age of 14 years were between the 10th and 20th centiles. The diagnosis was spondylometaphyseal dysplasia resembling type Kozlowski.

Family K
The phenotype of the proband has been previously described.\(^{29}\) Briefly, the proband presented at the age of 2 years with proportionate short stature (79 cm, <3rd centile) lumbar lordosis, limited joint mobility of hips, varus deformity of the lower limbs and a discrete waddling gait. Radiographs showed that the proximal femoral metaphyses were irregular and enlarged. The diagnosis was metaphyseal anadysplasia.

Family L
The proband presented as an active boy of 8 years of age with normal stature and without complaints. He had a varus deformity of his
Mutations within the gene encoding the α1(X) chain of type X collagen (COL10A1)

Mutations within gene encoding height

At the age of vertebrae lesions and showed that she had severe childhood this time wings.

0 showed radiographs The retardation that mildly only was considered.

diagnosis of autosomal affected. The brothers had an older unaffected sister and their parents were normal and non-consanguineous. Inheritance was apparently recessive but could be autosomal dominant or X linked. The diagnosis was spondylometaphyseal dysplasia.

Family M

The proband presented at birth with short limbs. He had generalised joint laxity, with anterior cruciate ligament absence and marked genu recurvatum. At the end of growth, he was of marked short stature having reached a height of 130 cm. His radiographs resembled those of hypochondroplasia (disproportionately short limbs, slight splaying of the metaphyses, and a long fibula) but he had, in addition, platyspondyly. The proband's younger brother was similarly affected with the addition of right sided hypoplasia and a small pinna. The brothers had an older unaffected sister and their parents were normal and non-consanguineous. Inheritance was apparently recessive but could be autosomal dominant or X linked. The diagnosis was spondylometaphyseal dysplasia.

Family N

A sister and brother were examined at the ages of 18 and 12 years, respectively, and found to have a form of short trunk short stature. Radiographs of the sister showed flattening and irregularity of all the vertebral bodies and metaphyseal lesions were evident at the hip and lower end of the ulna, but the epiphyses were normal. She had attained a height of 120 cm. In addition, she had a bulging forehead and slight exophthalmos but no other deformities. The brother had similar short stature but not as marked as his sister and radiographs showed his hips to be normal but with a metaphyseal lesion at the wrist. He, too, had irregular platyspondyly like his sister, as well as the bulging forehead. Their parents were unaffected. The diagnosis was considered to be an unspecified form of spondylometaphyseal chondrodysplasia of autosomal recessive inheritance. The diagnosis was spondylometaphyseal dysplasia.

Family O

The proband was first examined at the age of 13 months because of proportionate growth retardation that had been noted at birth. Her radiographs showed major irregularities of the femoral head whereas other metaphyses were only mildly affected. The height of the iliac wings was reduced and an ovoid aspect of the vertebrae was observed on a lateral micrograph. At the age of 4 years her height was 91 cm and at this time radiographs showed metaphyseal lesions and shortness of the femoral neck. The proband's mother had an adult height of 142 cm. Radiographs of the mother during childhood showed that she had severe coxa vara associated with a slight reduction in the height of the vertebral bodies. She had an osteotomy to correct the hip deformity. The proband's brother was similarly affected. The diagnosis was spondylometaphyseal dysplasia type Sutcliffe.

PCR and SSP analysis

The coding regions, intron/exon splice junctions, and 5' non-coding sequence extending from nucleotide -274 (the transcription start site has been designated +1)31, including exon 1 and 51 bp of intron of the COL10A1 gene, were amplified in six fragments (fragments A to F) by the PCR reaction from samples of genomic DNA, and analysed using the technique of SSCP as previously described.29,30 In addition, a 678 bp PCR fragment (the G fragment), including 74 bp of exon 1, intron 1, and 57 bp of exon 2, was similarly analysed by SSCP before and after digestion with the restriction endonuclease Aul and double digestion with SfAlI and XhoII. The oligonucleotide primer sequences for the amplification of this fragment were 5' GCACTGCTGATCTGGCCAG 3' and 5' GTTCAAGGATCCCTAGACAGC 3'. The PCR conditions were 94°C for 30 seconds, 60°C for two minutes and 72°C for one minute for 35 cycles. Duplicate denatured and non-denatured samples were loaded for all fragments on the SSCP gels. Heteroduplexes were observed in the non-denatured sample tracks and in the denatured sample tracks, where residual double stranded material remained.

Sequencing of DNA fragments

PCR fragments (either fragments B or E) which had an abnormal mobility following SSCP analysis were reamplified by PCR (using the 5'B and 3'B or 5'E and 3'E primer pairs)30 and purified from 1% agarose gels using the GeneClean II kit (Bio 101 Inc). For direct sequence analysis of the region of the gene encoding the entire NC1 domain, DNA was amplified using a primer located in the triple helix and a primer in the 3' untranslated region (primers 5'B and 3'E, respectively). The PCR conditions were 94°C for 30 seconds, 65°C for two minutes, and 72°C for one minute for 35 cycles. Purified DNA was either sequenced directly using γ-32P ATP end labelled primers and the SequiTherm Cycle Sequencing Kit (Cambio Ltd) or following cloning into the TA vector (TA Cloning Kit, Invitrogen) or M13mp18 (Boehringer-Mannheim). Single stranded DNA prepared from M13mp18 clones was sequenced using complementary primers and the Sequenase sequencing system (United States Biochemical).

Detection of polymorphisms

COL10A1 associated polymorphisms 1 to 7 were detected using PCR and restriction site generating PCR followed by restriction analysis as previously described.32 The polymorphisms numbered 1 to 5 are as described in Rash et al33 and are the Bi, Bii, Eii, Ei, and D polymorphisms, respectively. Those that were described in Sweetman et al34 Polymorphism 6 alters a Del1 site within the 5' untranslated
region of the gene (nucleotide position –60) and polymorphism 7 alters a HindIII site within a 1.4 kb PstI/EcoRI fragment located approximately 300 bp from the 3’ end of the gene. Restriction endonucleases were purchased from Boehringer-Mannheim and used with the buffers provided according to the manufacturers’ instructions. The products of restriction digestion were separated on 6% polyacrylamide gels and stained with ethidium bromide.

Results

IDENTIFICATION OF NOVEL SSCP CHANGES

Fragments A to G that spanned the three exons, intron 1, intron/exon splice junctions, 274 bp of 5’ untranslated sequence, and 59 bp of 3’ untranslated sequence of the COL10A1 gene were amplified from genomic DNA obtained from each of the probands from families A to O (fig 2). Fragments A, B, C, F, and G were restricted with combinations of restriction enzymes and all fragments were analysed on two SSCP gels which were either electrophoresed at 55 W for three to four hours at 4°C or at 3 W for 12 to 16 hours at room temperature. Using this approach, SSCP changes and heteroduplexes that did not result from the presence of previously identified polymorphisms or sequence variants were identified within the COL10A1 gene of the probands from families A, B, C, and D (fig 3). The SSCP changes and heteroduplexes were in each instance located to the region of the gene encoding the NC1 domain of 21(X) (nucleotides 1654–2136 and amino acids 520–680) and were present in other affected members of the families but absent in unaffected family members (fig 3). The migration changes were more precisely located to the E fragment (nucleotides 1933–2204) of affected members of families A and C and to the 243 bp AluI digestion product (nucleotides 1777–2019) of the B fragment (nucleotides 1218–2019) of affected family members from families B and D.

SEQUENCE ANALYSIS OF FOUR NOVEL MUTATIONS WITHIN THE NC1 DOMAIN OF THE 21(X) CHAIN

Sequence analysis was used to identify the mutations of the probands of families A to D (fig 4). From double stranded sequencing in both the 5’ and 3’ direction, proband C3 was found to be heterozygous for the deletion of the nucleotide T at position 1956, which would alter the codon for the proline at position 620 from CCT to CCG and introduce a premature stop codon at the following amino acid, position 621. It was necessary to clone the PCR amplified DNA from probands A3, B4, and D4 to determine the precise nature and extent of their mutations. Proband A3 was found to be heterozygous for the deletion of two base pairs, AC, at nucleotide positions 2029–2030, which would alter the reading frame from amino acid 645 and introduce a premature termination codon at amino acid position 647. Proband B4 was found to be heterozygous for the deletion of four base pairs, ACTT at nucleotide positions 1864–1867, which would alter the reading frame from amino acid 590 and introduce a premature termination codon at amino acid position 604. Proband D4 was found to be heterozygous for a single base pair transition of T to C at nucleotide position 1994 which would lead to the substitution of a serine for a proline at amino acid position 600. Concurrent sequence analysis of the region of the COL10A1 gene encoding the entire NC1 domain of the proband E3 did not show any novel sequence changes.

SEGREGATION ANALYSIS OF COL10A1 POLYMORPHISMS

The COL10A1 genotypes of members of families E, F, M, N, and O were determined for previously identified polymorphisms. The polymorphisms segregated with the phenotype in families F and O, were concordant with the phenotype in family M, were non-informative in family E, and were discordant with the phenotype in family N (fig 2).
Mutations within the gene encoding the α1(X) chain of type X collagen (COL10A1)

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Discussion
We have identified a further four mutations that cause MCDS. A total of 21 different mutations causing this condition have now been identified (table) and in each instance these mutations occur in the region of the gene encoding the NC1 domain (amino acids 520–680) of the α1(X) chain. Further, the mutations that have been identified so far cluster in the more carboxy-terminal half of this domain with the most 5' mutation altering the reading frame of amino acid position 590 and the most 3' mutation introducing a premature termination signal at amino acid position 665. This latter mutation causes the truncation of the chain by only 16 amino acids (that is, one tenth of the NC1 domain). In all instances, the mutations result in the same phenotype indicating that, irrespective of the nature of the mutation, the molecular consequences are likely to be the same. We were unable to identify COL10A1 mutations in the affected subjects from one family with classic features of MCDS despite sequence analysis of the NC1 encoding domain and detailed analysis of the other regions of the gene. Other researchers have also been unable to find COL10A1 mutations in certain families with MCDS despite similar analyses. The apparent absence of mutations in some subjects with MCDS may be a consequence of the SSCP technique not being 100% efficient in the detection of mutations or a second locus may be responsible for this condition. Conclusive evidence for an alternate locus has, however, not been obtained as negative linkage between the COL10A1 locus and the MCDS phenotype has not been shown. We, and others, have also not identified COL10A1 mutations in other people with forms of metaphyseal and spondylometaphyseal chondrodysplasia.

The finding of mutations within the region of COL10A1 encoding the NC1 domain has previously led to the hypothesis that these mutations would lead to the synthesis of chains of altered structure which would prevent their association into type X collagen trimers.
According to this hypothesis, exclusion of the abnormal chains from type X collagen trimers would result in a 50% reduction of type X collagen in the matrix and the MCDS phenotype could therefore be explained in terms of haploinsufficiency. This haploinsufficiency model has been supported by in vitro studies carried out by Chan et al., who examined the assembly of normal type X collagen and type X collagen chains containing a G618V amino acid substitution within the NC1 domain produced in an in vitro transcription and translation system. They showed that normal expressed products were able to associate into stable structures via the NC1 domain whereas the products of abnormal sequence were not able to form stable associations. Further, the abnormal chains did not interfere with the association of the normal chains when the two sequences were coexpressed. On the basis of these results, the authors hypothesised that the MCDS phenotype results from the exclusion of the abnormal chains from type X collagen trimers and the subsequent intracellular degradation of the unincorporated chains.

Despite the in vitro evidence for haploinsufficiency being the basis for the MCDS phenotype, the restricted distribution of COL10A1 mutations causing MCDS argues strongly against this being the mutation mechanism. In particular, several classes of mutations within COL10A1 that could predictably reduce the level of normal type X collagen synthesised and deposited in the matrix have not been identified to cause MCDS. These mutations included upstream frameshift mutations, single base pair substitutions resulting in premature termination codons, promoter or enhancer mutations, or loss of an entire allele because of large deletions or rearrangements. Certain of these mutations within COL10A1 (the gene encoding the proα1(I) chain of type I procollagen) have, however, been found to cause osteogenesis imperfecta type I (OI1), the mildest form of this inherited bone disease, which has been likened to MCDS in that it is caused by production of half normal amounts of type I procollagen. A recent report of mutations that caused OI I described two single nucleotide substitutions that altered 5' donor splice sites, a point mutation within an exon that created a premature termination codon, and four small deletions that caused translational frameshifts and new termination codons downstream of the mutations sites.

This means, reduced steady state levels of mRNA from the mutant COL10A1 alleles were observed in both total and nuclear RNA extracts isolated from fibroblasts from affected family members such that the mutant COL10A1 alleles were effectively “null” alleles. Six of the mutations occurred in regions of the gene encoding the triple helical domain and one in the region of the gene encoding the telopeptide. The kinds of mutations causing OI type I should be detected readily using the methods that we and others have used to identify mutations within COL10A1 in persons from MCDS, yet similar mutations causing MCDS have not been found.

The phenotypes of the type X null mice and the type X transgenic mice also argue against haploinsufficiency as the mutation mechanism underlying MCDS. Mutant mice homozygous for the type X collagen null mutation were viable and fertile and had apparently normal long bone growth and development. In addition, the absence of type X did not appear to have any effect on mineralisation or the distribution of other ECM components (osteopontin, osteocalcin, and type II collagen). The absence of a phenotype in the type X null mice was a surprising finding and seriously questioned whether half normal amounts of type X collagen could produce the phenotype of persons with MCDS. Further, the absence of a phenotype in the type X null mice has also brought into doubt whether the phenotype of the type X collagen transgenic mice was the result of a dominant negative mutation mechanism as had been previously proposed. The type X collagen transgenic mice were generated by microinjection of mice embryos with chick type X collagen constructs that had internal in frame deletions of the triple helical encoding domain and a downstream chloramphenicol acetyltransferase gene. Transgenic mouse lines were established that had, to a greater or lesser extent, thoracolumbar kyphosis and growth retardation and, in a proportion of the more severely affected mice, the bone marrow showed a predominance of mature erythrocytes but a paucity of leucocytes, and the sizes of the thymus and spleen were reduced. Tissue specific expression of the transgenes was confirmed by northern blot analysis and immunohistochemistry, using monoclonal antibodies specific for chick type X collagen, and showed that the transgene product colocalised with regions showing histological defects. The precise mechanism whereby the transgene product caused the phenotype of the transgenic mice is
not known and interaction between the chick type X collagen chains and the endogenous type X collagen chains was not proven. A dominant-negative mutation mechanism implies that the chick chains interact with the endogenous mouse type X collagen chains and render them inactive by, for example, causing their rapid degradation within the cell. This process would then lead to a reduction in the amount of normal type X collagen deposited in the matrix and the extent of the reduction would depend on the level of expression of the transgene. As we now know that mice without any detectable type X collagen are phenotypically normal, coupled with the fact that the chick type X collagen of the transgenic mice was detected immunohistochemically within the matrix, it is more likely that the presence of the chick gene product had a dominant gain of function effect.

In the light of the genetic findings of the distribution of mutations causing MCDS and the studies of the type X null and transgenic mice, an alternative mutation mechanism other than that of haploinsufficiency must be responsible for the MCDS phenotype. We propose that a dominant gain of function mutation mechanism underlies this phenotype. For this to be true, either the abnormal type X collagen chains are able to associate with the normal chains and produce type X collagen trimers of altered structure that have a deleterious intracellular or extracellular effect or, as the in vitro data suggest, the abnormal chains are not able to associate into trimers, but their presence within the cell has a deleterious effect on the function of that cell.

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