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# Exclusion of the COL2A1 gene as the mutation site in diastrophic dysplasia

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SUMMARY The involvement of the cartilage specific type II collagen gene (COL2A1) was studied in nine patients with diastrophic dysplasia in the Finnish population, where the prevalence of this chondrodystrophy clearly exceeds that reported for other populations. COL2A1 was chosen as the candidate gene based on previous morphological and chemical studies which suggested abnormal structure of type II collagen in diastrophic dysplasia. Southern analysis of the patients' DNA showed no disease related differences in any of the restriction fragments covering the 30 kb COL2A1 gene. As a second approach, the nine patients and their 74 relatives were studied for the inheritance of the type II collagen gene. Three of the patients with diastrophic dysplasia were not homozygous for the intragenic RFLP markers, which suggests that the disease is not linked to the type II collagen gene. Multipoint linkage analysis gave a lod score of −2·95, which conclusively excluded the COL2A1 gene as the mutation site in diastrophic dysplasia in these families.

Diastrophic dysplasia is an autosomal recessive form of short limbed dwarfism, which has been well characterised clinically and radiologically.[1–4] The disease occurs with a relatively high frequency (at least 1:35 000) in the Finnish population and thus its prevalence clearly exceeds that reported for other populations.[4] Such a high prevalence could be explained by the founder effect and geographical isolation; in such cases the affected subjects should have common ancestors and be homozygous for the disease gene and intragenic RFLP markers. Protein chemistry studies have suggested the type II collagen gene (COL2A1) as the mutant locus in diastrophic dysplasia; an abnormality near the collagenase cleavage site of the type II collagen was observed in the SLS (segment long spacing) banding pattern of the protein in three patients with diastrophic dysplasia.[5 6] Detailed characterisation of the human COL2A1 gene[7–10] on chromosome 12 has made molecular genetic studies in the chondrodysplasias possible.[11 12]

In this paper we report the results of both structural gene analyses and linkage studies used to determine the possible role of the type II collagen gene in diastrophic dysplasia.

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## Materials and methods

### PATIENTS

The patients were either referred to the Genetics Clinic of the Children's Hospital, Helsinki for diagnostic studies and orthopaedic evaluation, or encountered at genetic consultations at the Orthopaedic Hospital of the Invalid Foundation, Helsinki. Nine patients, six females and three males, from seven families were included in the study. The age range was three months to 41 years. The diagnosis of diastrophic dysplasia was based on clinical and radiographical characteristics.[2 3] Blood samples were collected after informed consent from the nine patients and their 74 unaffected relatives as shown in the pedigrees.

### METHODS

DNA was prepared from heparinised or EDTA treated peripheral blood, digested with restriction endonucleases, and electrophoresed on 0·75 to 1·5% agarose gels using standard methods.[13] Transfer by blotting onto Hybond-N membranes

(Amersham International, Amersham, UK) and hybridisations were performed as suggested by the supplier of the membrane, using nick translated $^{32}$P-dCTP labelled probes.

Both the cosmid clone cosHcol1,[8] covering the whole proα1(II) collagen gene, and genomic subclones[7 14] were used as COL2A1 specific probes in Southern hybridisations as described previously.[13] The additional probes used to detect RFLPs on chromosome 12 are listed in the table.

Multipoint linkage analyses were performed using the LINKAGE program.[17] LIPED[18] was used for calculation of linkage between the disease and individual markers.

## Results

### ANALYSIS OF THE GENE NEAR THE COLLAGENASE CLEAVAGE SITE

In order to correlate the protein structure (amino acid sequence and SLS banding pattern) with the gene structure (exon-intron organisation and restriction sites) of type II collagen near the vertebral collagenase cleavage site, a composite map was constructed (fig 1). The information available on the structure of the human type II collagen gene included restriction sites[12 13] and the location and nucleotide sequences of a number of exons.[8–10] These data were combined with the amino acid sequence of the triple helical collagen molecule[10] and with the SLS banding pattern.[19 20] This information was used to determine the location of genomic sequences corresponding to the observed structural alteration of type II collagen protein near the collagenase cleavage site (SLS bands 41 to 45).[5 6]

For these studies, DNA was isolated from nine patients with diastrophic dysplasia, from their unaffected sibs, and from healthy controls and analysed by single and double digestions with several restriction enzymes followed by Southern analysis of

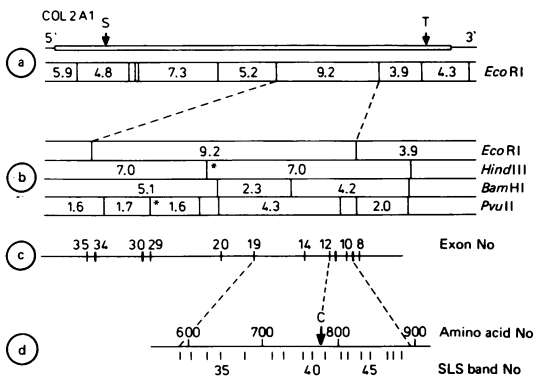


FIG 1 *Correlation of the protein and gene maps of human type II collagen. The figure shows from top to bottom: (a) The overall structure of the COL2A1 gene with the sizes of EcoRI restriction fragments. The arrow marked S shows transcription start site, the arrow marked T shows the polyadenylation site. (b) Partial restriction map of the central part of the gene. (c) The exon-intron organisation of the gene in scale with the restriction map. The exons are numbered from the 3' end. (d) Partial SLS banding pattern and the corresponding amino acids (numbered for the triple helical domain). The arrow marked C shows the cleavage site of the vertebrate collagenase.*

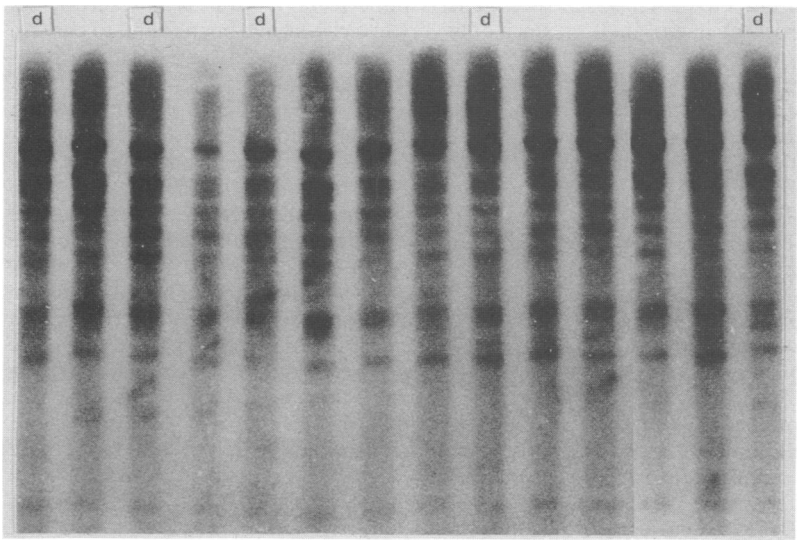


FIG 2 *Analysis of PvuII restriction sites by Southern hybridisation of DNA from nine diastrophic subjects and nine unaffected sibs. Aliquots of DNA (5 μg) were digested with PvuII, electrophoresed on 1·25% agarose gels, and transferred by blotting to Hybond-N membrane. Lanes marked d represent diastrophic patients, unmarked lanes represent unaffected subjects. The filter was hybridised using genomic subclones covering the region of the gene coding for the triple helical domain.*

TABLE *The polymorphic DNA markers on chromosome 12 used in the study.*

| *Locus* | *Probe* | *Restriction enzyme* | *Frequency of allele* | *Reference* |
|---|---|---|---|---|
| *COL2A1* | pKEV4 | *Pvu*II | +0·46<br>−0·54 | 13 |
| | cosHcol1 | *Hind*III | +0·33<br>−0·67 | 13 |
| *D12S14* | pEFD33·2 | *Taq*I | Unknown | 15 |
| | | *Msp*I (a) 9·0 kb | 0·41 | 15 |
| | | (b) 7·0 kb | 0·39 | |
| | | (c) 4·3 kb | 0·05 | |
| | | (d) 3·2 kb | 0·15 | |
| *D12S4* | p9F11 | *Taq*I | +0·36<br>−0·64 | 16 |
| *D12S8* | p7G11 | *Msp*I | +0·27<br>−0·73 | 16 |

the fragment sizes. Apart from the polymorphic restriction fragments no differences in the migration of the DNA bands could be detected between the samples, as shown in fig 2.

LINKAGE STUDIES

The patients and their pedigrees were studied in two groups for the inheritance of the COL2A1 gene and other RFLP markers on chromosome 12 (table). A genealogical study on population records maintained by the Lutheran church back to the 16th century indicated that four of the families were related (fig 3). These four families were studied in the first group (fig 4). Probably because of the considerable inbreeding, a high degree of homozygosity was observed for each marker; the *Bam*HI RFLP within COL2A1 and the flanking *Taq*I RFLP detected with probe p9F11 were completely uninformative. Homozygosity for the absence of both the *Pvu*II and *Hin*dIII sites within COL2A1 was clearly overrepresented in the present material. In the Finnish population the frequency for the absence of the *Hin*dIII site is 0·67, that for the absence of the *Pvu*II site 0·54, and for the *Bam*HI site 0·96.[13] In the present study, the corresponding frequencies for all the subjects studied were 0·79 for *Hin*dIII and 0·77 for *Pvu*II. Of the other chromosome 12 markers, the *Msp*I four allele polymorphism and the *Taq*I dimorphism detected by probe pEFD33·2 were the most informative ones (fig 4). These results clearly showed that the disease was not linked to the *D12S14* locus. The information obtained with probe p7G11 detecting another *Msp*I dimorphism was less informative, but also pointed towards exclusion.

The other three families with diastrophic children were studied using the same probes (fig 5). One family with three diastrophic children (pedigree 5a) was found to be completely uninformative with respect to all the three COL2A1 markers. Again the pEFD33·2 probe detecting the *D12S14* locus turned out to be highly informative. Combining all the available data on the nine patients indicated that three of them were not homozygous for the three intragenic markers.

Multipoint analysis using the LINKAGE computer program was used to calculate linkage between diastrophic dysplasia and RFLP markers within COL2A1 and the flanking *D12S14* locus in pedigrees 4a to d and 5a without taking the consanguinity into account. The analysis gave a lod score of −2·95 between the disease and COL2A1 loci at recombination fraction 0. For these analyses the distance between the two marker loci was given the recombination fraction value of 0·1, which was obtained using the LIPED program.

## Discussion

Attempts to determine the molecular basis of the chondrodysplasias have met with the difficulty of isolating abnormal molecules from the cartilage matrix. Direct analysis of the genome using molecular biological techniques should therefore be particularly suitable for studies on heritable cartilage diseases. Diastrophic dysplasia is one of the diseases where protein chemistry has suggested involvement of the COL2A1 gene,[5 6] although confirming reports from other laboratories are not available, except for a recent segregation analysis showing weak linkage of COL2A1 to diastrophic dysplasia in one pedigree.[12] In the present study we did not find any disease related gross rearrangements or alterations in fragment lengths or restriction sites of the type II collagen gene. In the light of current knowledge about mutations of type I collagen in osteogenesis imperfecta[21 22] this is not surprising, since only very few cases have been found to contain deletions large enough to be detected with conventional Southern hybridisation.

Since such results could not exclude a mutation in the proα1(II) collagen gene in diastrophic dysplasia, another approach using linkage analysis was also used. The parents in pedigrees 4b, 4c, and 4d and the mother of the patient in pedigree 4a were found to be consanguineous (fig 3). In the light of the high prevalence of diastrophic dysplasia in the relatively isolated Finnish population and the relatively recent common ancestors in the four sibships one would expect all the affected subjects to have the same homozygous haplotype of the affected gene. Using two RFLPs within the COL2A1 gene we were able to show that this was not the case. One of the four subjects in the large pedigree did not fulfil these expectations. Two of the other five patients were also found to be heterozygous for the RFLP markers within the COL2A1 locus.

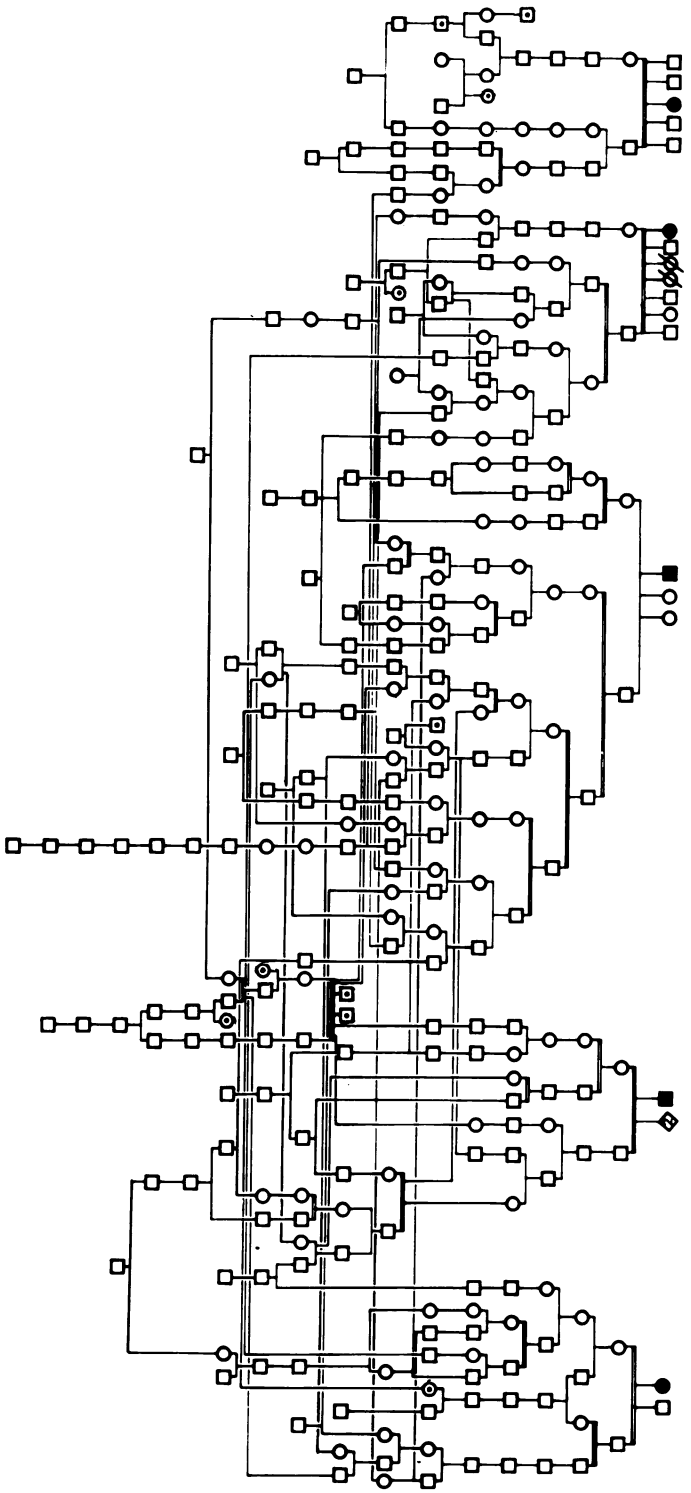


FIG 3 *Pedigree of Finnish diastrophic dysplasia families. Patients who were crippled according to church records are marked with a dot. The families marked a to d correspond to those shown in fig 4.*

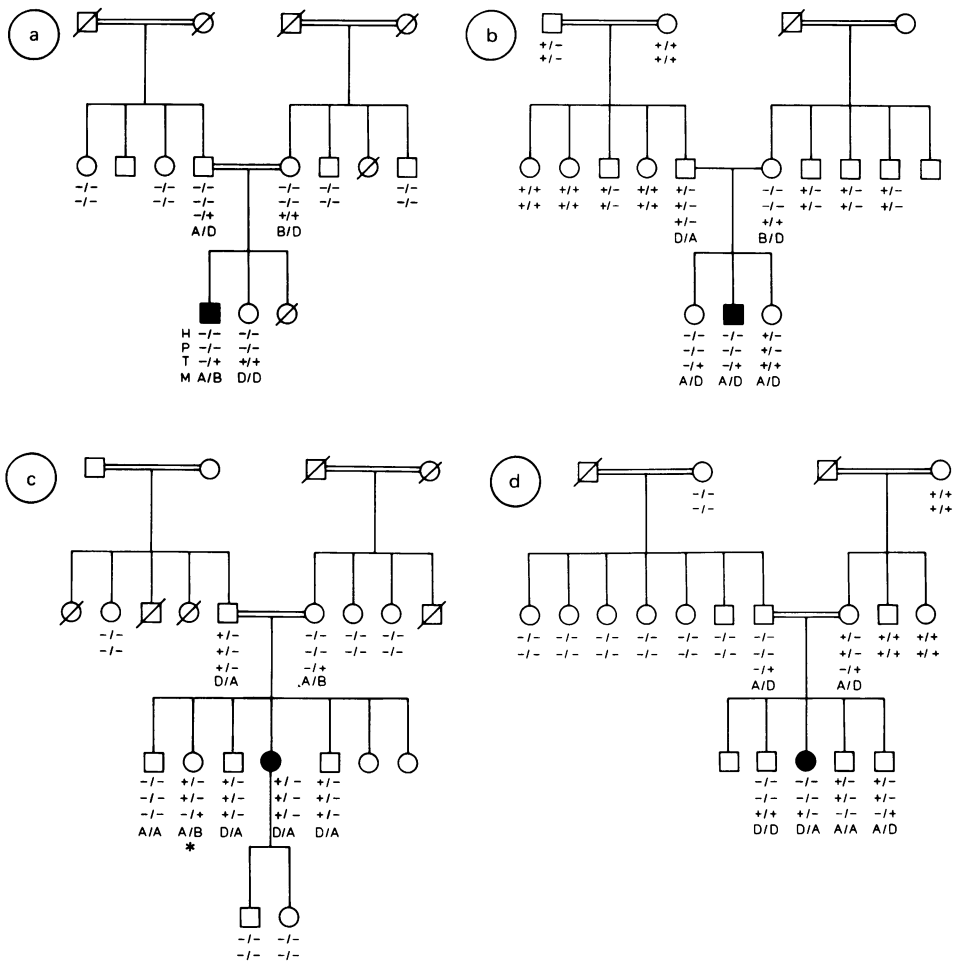


FIG 4 *Genotype analysis of the four consanguineous pedigrees shown in fig 3. The genotypes refer to the four markers used: H=HindIII dimorphism, P=PvuII dimorphism, T=TaqI dimorphism recognised by pEFD33·2, and M=MspI four allele polymorphism recognised by pEFD33·2 (see table). *Subject with a recombination between the COL2A1 and D12S14 loci.*

Further proof for the exclusion of COL2A1 and nearby loci on chromosome 12 came from the use of other RFLP markers (figs 4 and 5). Initially two point analyses between the disease and each of the informative marker loci were performed, followed by multipoint analysis to maximise the information. Analysis of pedigrees 4a to d and 5a gave a negative lod score of 2·95, which confirms our hypothesis, that is, excludes the COL2A1 locus as the mutation site in diastrophic dysplasia.

The most important conclusion of the present study is to direct attention to a search for candidate genes other than COL2A1 in diastrophic dysplasia. Assuming a common source for all the nine affected Finnish subjects, the study can be continued by a random search for homozygosity using the large selection of other polymorphic markers available.

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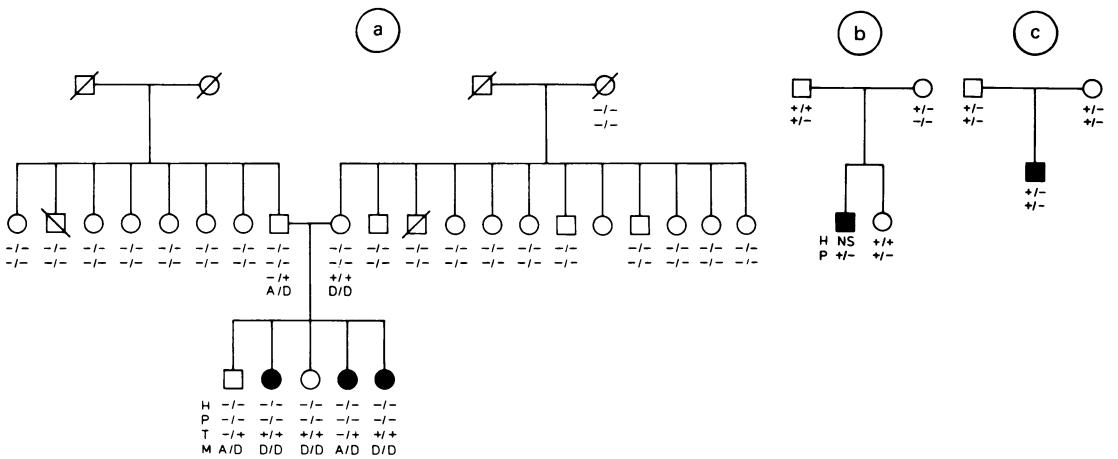


FIG 5 *Genotype analysis of the three other Finnish families with diastrophic children. The genotypes are marked as in fig 4.*

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