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An exon skipping mutation of a type V collagen gene (COL5A1) in Ehlers-Danlos syndrome

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Abstract

The Ehlers-Danlos syndrome (EDS) is a heterogeneous group of inherited connective tissue disorders characterised by skin hyperextensibility, joint hypermobility, easy bruising, and cutaneous fragility. Nine discrete clinical subtypes have been classified. We have investigated the molecular defect in a patient with clinical features of Ehlers-Danlos syndromes types I/II and VII. Electron microscopy of skin tissue indicated abnormal collagen fibrillogenesis with longitudinal sections showing a marked disruption of fibril packing giving very irregular outlines to transverse sections. Analysis of the collagens produced by cultured fibroblasts showed that the type V collagen had a population of α1(V) chains shorter than normal. Peptide mapping suggested a deletion within the triple helical domain. RT-PCR amplification of mRNA covering the whole of this domain of COL5A1 showed a deletion of 54 bp. Although six Gly-X-Y triplets were lost, the essential triplet amino acid sequence and C-propeptide structure were maintained allowing mutant protein chains to be incorporated into triple helices. Genomic DNA analysis identified a de novo $G^{+3} \rightarrow T$ transversion in a 5′ splice site of one COL5A1 allele. This mutation is analogous to mutations causing exon skipping in the major collagen genes, COL1A1, COL1A2, and COL3A1, identified in several cases of osteogenesis imperfecta and EDS type IV. These observations support the hypothesis that type V, although quantitatively a minor collagen, has a critical role in the formation of the fibrillar collagen matrix.

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Key words: Ehlers-Danlos syndrome; type V collagen (COL5A1); exon skipping.

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Ehlers-Danlos syndrome (EDS) is a group of inherited connective tissue disorders whose primary clinical features are skin hyperextensibility, joint hypermobility, easy bruising, and cutaneous fragility. The Berlin Nosology[1] recognises nine distinct clinical subtypes. For some of these the molecular nosology is well established. Thus, abnormalities of type III collagen cause the vascular fragility associated with EDS type IV[2] and defects in the modifying enzyme lysylhydroxylase lead to EDS VI.[3] In EDS VII a failure to convert type I procollagen to collagen owing to structural abnormalities of the α1(I) or α2(I) chains[3] or a deficiency in the processing enzyme, procollagen-N-proteinase,[4–6] results in extreme joint laxity and skin fragility. However, the molecular bases of the two most common forms, EDS I and II, have not been identified. Abnormalities of collagen fibril architecture observed in these subtypes[7] strongly suggests an underlying disturbance of collagen fibrillogenesis. However, linkage studies in a few EDS I and II families have excluded several major fibrillar collagens as candidate genes[8–10] leaving minor components of the fibrils such as type V, type XII, or type XIV collagens as potential candidates. Recently linkage to COL5A1 has been reported in one large EDS II kindred[11] although discordant segregation has also been reported.[12] Additional recent studies in our laboratory have identified further families linked to COL5A1 (Burrows *et al*, in press, Burrows *et al*, in preparation).

Type V collagen is a low abundance fibrillar collagen with a wide distribution. It occurs in such diverse tissues as fetal membranes, placenta, skin, bone, cartilage, tendon, cornea, synovial membrane, blood vessel walls, liver, and lung. Three distinct α chains have been identified as components of type V collagen; $\alpha1(V)_2\alpha2(V)$ appears to be the most abundant molecular species but $\alpha1(V)_3$ and $\alpha1(V)\alpha2(V)\alpha3(V)$ molecules may also exist in certain tissues.[13] The cDNAs for the human α1(V) and α2(V) chains have been cloned and sequenced[14–18] and their structural genes localised to human chromosomes 9q34.3 for COL5A1[19,20] and 2q24.3–q31 for COL5A2.[21]

Here we present the biochemical and molecular characterisation of a type V collagen defect in a young woman with atypical EDS II. She produces both normal and shortened α1(V) chains with some carrying a deletion in the triple helical domain as a result of skipping a 54 bp exon in the mRNA transcripts because of a point mutation in one COL5A1 allele. We believe this represents the first characterisation of a naturally occurring structural mutation in a human type V collagen gene.

Materials and methods

CASE REPORT

Clinical examination of the proband, a 24 year old woman, showed generalised skin fragility with extensive scarring of the forehead, shins, and knees and scattered bruising on the arms and legs. There was a marked generalised joint laxity with severe premature bilateral hallux valgus and diamond shaped feet. She was short (155 cm) with a mild thoracic kyphoscoliosis,

pectus excavatum, and audible mitral valve prolapse. The eyes were unusually prominent and ophthalmological examination showed a hypermetropia caused by flattened corneas. She suffered a miscarriage 10 weeks into her first pregnancy but a second went to term and a normal baby delivered. Family history was negative, she was the third of six children, and all sibs and both parents were clinically normal. Skin biopsies from the inner aspect of the upper arm and blood samples were obtained from the proband and both parents. Subsequently, a second skin sample was obtained from the proband's toe during corrective surgery of a fixed flexion deformity.

Because of the short stature and scoliosis the differential diagnosis in this patient initially favoured EDS VII, but the cutaneous fragility and other features were also consistent with EDS types I and II.

ELECTRON MICROSCOPY

Skin samples (1 mm^3) were fixed by immersion in 2.5% glutaraldehyde in 0.1 mol/l sodium cacodylate buffer, pH 7.2, containing 3 mmol/l $CaCl_2$ for two to four hours. They were subsequently washed in sodium cacodylate buffer, pH 7.2, with 3 mmol/l $CaCl_2$, postfixed for 30 minutes in 0.05% osmium tetroxide in buffer plus calcium, and then en bloc stained in 2% aqueous uranyl acetate for 30 minutes. The tissues were dehydrated through an ascending series of ethanols to propylene oxide and were finally embedded in araldite CY212. Sections of approximately 70 nm thickness were cut, mounted on copper grids, and stained with uranyl acetate and lead citrate before examination in the electron microscope.

PROTEIN STUDIES

Skin fibroblasts were labelled with ^{14}C-proline (1 μCi/ml).[22] Proteins were harvested from the medium by ethanol precipitation and resuspended in 0.5 mol/l acetic acid. The washed cell layers were scraped into phosphate buffered saline (PBS), pelleted by low speed centrifugation, then lysed by suspension in 0.5 mol/l acetic acid containing 0.5% triton X100 at 4°C. The extracts were clarified by centrifugation (13000 *g*, 10 minutes, 4°C) and supernatants retained. Medium and cell layer procollagens were converted to collagens by limited pepsin digestion of native protein before analysis.[22] In some instances 0.05% dextran sulphate was added to the labelling medium to induce processing of precursors.[23] In this case ethanol precipitates of the medium were redissolved in 0.1 mol/l NH_4HCO_3 and cell layer pellets were solubilised by incubating with 0.1 mol/l NH_4HCO_3/0.1% SDS at 100°C for five minutes before clarification. The extract was then analysed without further treatment. Procollagen and collagen chains were analysed on 5% polyacrylamide gels containing 2 mol/l urea using the tris-glycine-SDS buffer system.[24] In situ cyanogen bromide peptide mapping was carried out essentially as described[25] using 10% polyacrylamide gels in the second dimension.

cDNA STUDIES

Cytoplasmic RNA was isolated by the NP40 lysis technique,[26] reverse transcribed, and PCR amplified to yield four overlapping fragments encoding the whole of the triple helical domain of the α1(V) chain using the following primers.

Fragment 1
COL5A1-Ex6S 5′-CAAGCCATTCTCCAGCAGGCCA-3′
COL5A1-972A 5′-CCTTTGGTCCTTGTCTTCCTGGAT-3′
Fragment 2
COL5A1-686S 5′-CTACCCAGGTCCTCGAGGAGTCAA-3′
COL5A1-1553A 5′-ATTGCCTTTCAGTCCAAGAGCTCC-3′
Fragment 3
COL5A1-1415S 5′-CCTTGCTGGAAAAGAAGGGACGAA-3′
COL5A1-2168A 5′-CACTGCACCAGGGTTTCCTATTCC-3′
Fragment 4
COL5A1-1956S 5′-CAGAAAGGTGATGAAGGTCCCAGAG-3′
COL5A1-3138A 5′-CGTAGTCCACGTAGTTCTCGCCATT-3′

An annealing temperature of 62°C and an extension temperature of 72°C was used. Products were analysed on agarose or polyacrylamide gels. Restriction enzyme digestions followed the suppliers' recommended conditions. Subcloning of a *Pst*I-*Nco*I subfragment of fragment 4 for sequencing used the M13 derivatives BM20 and BM21 (Boehringer–Mannheim) which contain an *Nco*I site in the polylinker. Sequencing was performed with a Sequenase v2.0 kit using the M13 universal primer or COL5A1-2339A (below) as sequencing primers.

GENOMIC DNA

Genomic DNA from the proband was PCR amplified using the primers COL5A1-2101S: 5′-CCTCCGGAGCTCCAGGTGCTGATG-3′ and COL5A1-2339A: 5′-AGGGCTGCC-TTTGGGACCATCATCT-3′ to yield a single 2.2 kb fragment. This was cloned into M13 directly or after restriction enzyme digestion and size fractionation of the fragments. Sequencing was performed using the M13 universal primer and Sequenase v2.0.

Allele specific oligonucleotides, ASO-A: 5′-CGCTCACTAACCGGGGG-3′ and ASO-C: 5′-CGCTCACTCACCGGGGG-3′ were end labelled with ^{32}P-γATP and T4 polynucleotide kinase and used to hybridise Southern blots of amplified genomic DNA from the proband, her parents, and several unrelated subjects. Filters were hybridised at 52°C in 6 × SSC, 5 × Denhardt's, 0.5% SDS, 10% PEG 8000 for 16 hours then washed to 1.5 × SSC at 52°C (ASO-C) or 2 × SSC at 52°C (ASO-A).

COL5A1 haplotype analyses were performed using the published primers.[27]

Results

ELECTRON MICROSCOPY

Electron micrographs of the patient's toe skin showed grossly abnormal collagen fibres. Transverse sections showed many fibres with a highly irregular profile (fig 1A, B). Longitudinal sections showed a marked disorganisation of fibril packing with the fibres splaying, twisting, and interweaving (fig 1C).The skin sample from the patient's upper arm also showed irregularly shaped fibres (fig 1D, E, F) but to

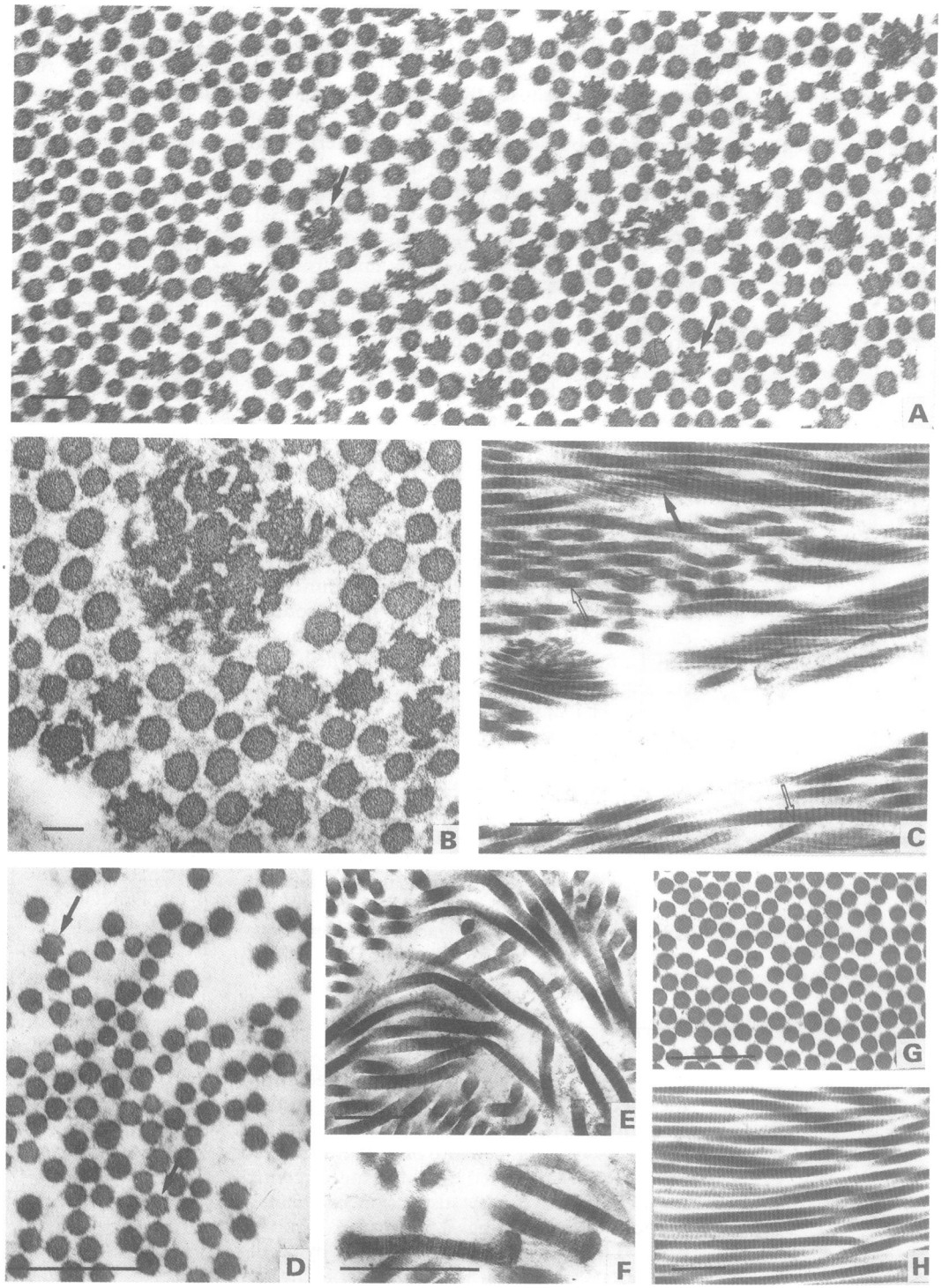

Figure 1 (A–C) Election micrographs of skin from the patient's toe taken at surgery. (A) Low power field in transverse section (TS) showing large numbers of collagen fibres with highly irregular profiles. Some fibres form larger arrays with "satellites" extending from the main fibre body producing a cog wheel effect (arrows). Scale bar = 0.2 μm. (B) Higher power TS of irregular fibres, some perhaps displaying fusion of smaller fibres to form a massively disordered fibre. Scale bar = 0.1 μm. (C) Collagen fibres in longitudinal section (LS) showing a disorganised layering and splaying of fibrils (bold arrows) and a tendency of fibres to basket weave (open arrows). (D–F) Electron micrographs through dermis from patient's inner forearm. (D) TS of collagen fibres showing some irregular profiles (arrows) but to a much lesser degree than the skin from the toe. Scale bar = 0.5 μm. (E) LS of collagen fibres showing marked angularity with sudden changes in direction. Scale bar = 0.5 μm. (F) LS of collagen fibres showing stub ends as the fibres abruptly change direction. Scale bar = 0.5 μm. (G, H) Electron micrographs showing the normal fibre structure and organisation in the dermis. (G) In TS the fibres are smooth and round and fairly regular in size. Scale bar = 0.5 μm. (H) In LS the fibres are closely packed with no angulation and displaying only a limited amount of weaving. Scale bar = 0.5 μm.

a much lesser degree. These differences may reflect the differing stresses experienced by the skin at these two sites. The normal appearance of collagen fibres in the skin shows highly regular round outlines with only limited interweaving (fig 1G, H).

PROTEIN ANALYSIS

SDS-polyacrylamide gel electrophoresis of medium proteins from the proband's cultured skin fibrobasts showed the normal range of processing intermediates for type I procollagen (not shown). There were no indications of

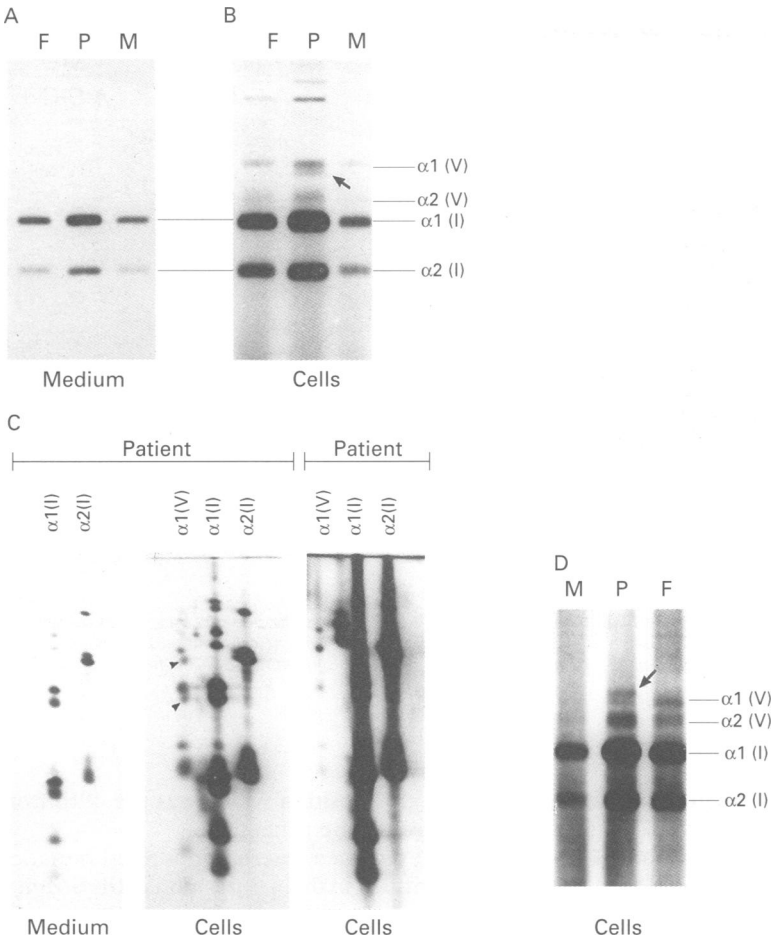

Figure 2 SDS-polyacrylamide gels of radiolabelled collagens from skin fibroblasts from the patient (P), her mother (M), and father (F). (A) Pepsin treated collagens from the culture medium. (B) Pepsin treated collagens from the patient's cell layer show a doublet in the α1(V) region (arrowed). (C) In situ cyanogen bromide peptide mapping of collagens from the patient's medium and cell layers. Double spots for some CB peptides (arrowheads) are observed in the patient's α1(V) chain. (D) Collagen chains isolated from the cell layer without proteolysis following incubation with dextran sulphate. The patient shows an additional higher molecular weight form of α1(V) (arrowed).

resistant pNα1(I) or pNα2(I) chains after pepsin (fig 2A), trypsin (not shown), or chymotrypsin (not shown) digestion of the procollagen. Pepsin digested collagens from the proband's cell layer showed a doublet in the α1(V) position with a novel component migrating just ahead of the normal α1(V) on SDS gels (fig 2B). This was not seen in either parent (fig 2B) nor in 20 unrelated EDS I/II patients or any control cell lines (not shown). In situ CNBr mapping (fig 2C) of the cell layer proteins indicated the novel component was an α1(V) derivative; double spots for some CB peptides (arrowheads, fig 2C) suggested a small deletion within the triple helical domain. Labelling the proband's cells in the presence of dextran sulphate to induce processing of procollagens showed α1(V) chains that were larger than normal (fig 2D) suggesting faulty processing of mutant molecules.

cDNA STUDIES

RT-PCR amplification of the triple helical domain of the α1(V) mRNA was achieved in four overlapping fragments using primers chosen from the published cDNA sequence.[14 15] The fragments were analysed by polyacrylamide gel electrophoresis (fig 3A). One fragment (COL5A1 1956-3138) from the proband's RNA yielded a doublet and characteristic heteroduplex bands, indicating a deletion, which did not occur in any controls or other EDS patients examined. Cloning and sequencing of a *Pst*I-*Nco*I subfragment spanning the deletion showed two sequences (fig 3B), one missing 54 bp compared to the other and the published sequences. The two published sequences[14 15] differ slightly in this region but the sequence obtained for the wild type clones here corresponded exactly to that of Greenspan *et al.*[15]

GENOMIC DNA STUDIES

When primers COL5A1-2101s and COL5A1-2339a flanking the deleted sequence were used to amplify the proband's genomic DNA, a single PCR fragment of approximately 2.2 kb was obtained. The whole product and selected restriction enzyme fragments were cloned into M13. Sequencing showed that the 54 bp deleted from the cDNA represented a discrete exon. Two sequences were observed just 3′ of this exon with either a G or a T (in the coding strand) at position +3 of the 5′ splice site (fig 4). Correlation of the sequences obtained here with the recently published intron/exon structure of COL5A1[28] identifies the deleted exon as the 49th exon from the 5′ end of the gene. Allele specific oligonucleotide (ASO) hybridisation of Southern blotted, amplified genomic DNA from the proband, her parents, and several controls identified the G^{+3} sequence in all samples, but the T^{+3} sequence occurred only in the proband's DNA (fig 5) implying a de novo mutation. Paternity was confirmed using highly polymorphic markers from chromosomes 1, 2, 6, 11, and 12. Subsequent testing by ASO hybridisation of the genomic DNA from the proband's clinically normal newborn infant showed it had not inherited the T^{+3} allele from its mother (data not shown). Further analysis using intragenic *Bst*UI and *Dpn*II polymorphisms[27] indicated that the mutation was in the proband's maternal COL5A1 allele (data not shown).

Discussion

The clinical features of this patient are consistent with a diagnosis of EDS types I/II or VII. The latter has been excluded on both biochemical and ultrastructural criteria. Firstly the skin fibroblast cultures showed neither abnormal processing of type I procollagen nor proteinase resistant pNα1(I) or pNα2(I) chains. Furthermore, the genomic DNA sequences around exon 6 of both COL1A1 and COL1A2, the usual sites for mutations in EDS VIIA or EDS VIIB, were shown to be normal. Secondly, the absence of characteristic hieroglyphic collagen fibrils in electron micrographs of the skin and a normal array of processing intermediates also excluded the newly recognised human enzyme deficiency of EDS VIIC.[4-6]

The doublet for the pepsinised α1(V) chains in the cell layer of fibroblast cultures suggested

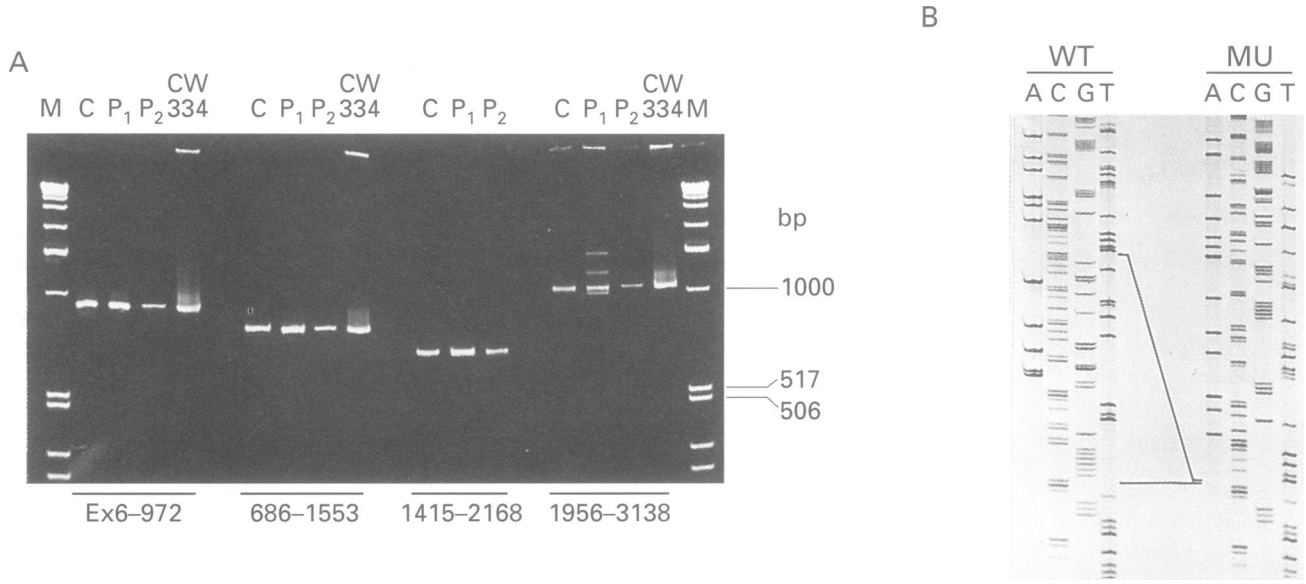

Figure 3 (A) Polyacrylamide gel of RT-PCR fragments covering the triple helical domain of the α1(V) chain. P_1 is the patient, P_2 is an unrelated EDS patient, C is a control cell line, CW 334 and CW 32 are cloned cDNAs for α1(V). M = 1 kb ladder. In the patient (P_1) the most 3′ fragment shows a doublet with heteroduplex bands. (B) Sequencing gel of mutant and wild type antisense clones of PCR fragment 1956-3138 from the patient showing 54 bp deletion.

heterozygosity for a small deletion within the triple helical domain of this protein. This was confirmed by RT-PCR amplification of this domain which showed a 54 bp deletion corresponding to six Gly-X-Y triplets. Although 54 bp is a common size for exons in collagen genes, homology of COL5A1 with other fibrillar collagen genes would have placed this deletion within the 162 bp exon 40. However, the genomic DNA analysis showed the deleted 54 bp represented a discrete exon in COL5A1. A recent characterisation of the complete intron/exon arrangement of COL5A1 has shown that it is far larger and more complex than other fibrillar collagen genes having 66 exons compared to 52.[28] It confirmed a 54 bp exon in this position which was the 49th from the 5′ end of the gene.

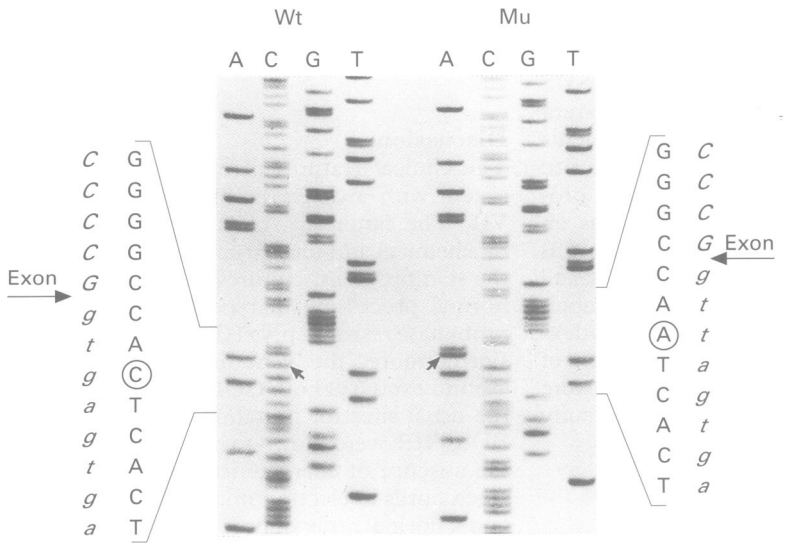

Figure 4 Sequencing gel of mutant and wild type antisense clones of genomic DNA from the patient. Small arrows indicate the single base mutation (C/A). Larger arrows indicate exon/intron boundary. Bases in upper case in complementary strand are exonic, lower case bases are intronic.

In our patient a heterozygous point mutation was identified in the +3 position of the 5′ donor splice site ($G^{+3}\rightarrow T$) of the intron following the deleted exon. The +3 position of a splice site is highly conserved, being A or G in 95% of mammalian 5′ splice junctions.[29 30] Thus exon skipping or alternative splicing would be anticipated from a mutation at this point. There was no evidence in the RT-PCR products to indicate the use of cryptic splice sites. Analogous exon skipping mutations in the major fibrillar collagens, COL1A1, COL1A2, and COL3A1, have been identified as the cause in several cases of osteogenesis imperfecta or Ehlers-Danlos syndrome type IV. In common with exon skips in these other fibrillar collagen genes the loss of the COL5A1 exon deletes a whole number of Gly-X-Y triplets while leaving the mRNA in frame. This maintains the essential triplet amino acid sequence motif and a functional C-propeptide which allows mutant protein chains to associate with other proα(V) chains (both normal and mutant) to form triple helical molecules. The conversion from procollagen to collagen in deletion mutant molecules may be hindered by misalignment of the N-propeptidase cleavage site and this was suggested in this case by the dextran sulphate labelling experiments yielding larger than normal α1(V) chains. Since the α1(V) chain is a component of all forms of type V collagen ($\alpha1(V)_2\alpha2(V)$, $\alpha1(V)_3$, and α1(V) α2(V) α3(V)) a heterozygous mutation in this chain would adversely affect the majority of type V collagen molecules.

Despite its low abundance type V has a very wide tissue distribution. It has been shown to coaggregate with type I collagen to produce heterotypic fibrils in many tissues.[31 32] It is

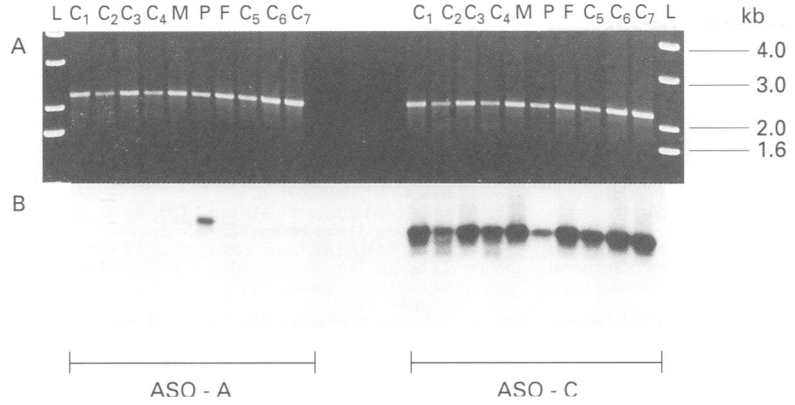

Figure 5 ASO hybridisation of PCR amplified genomic DNA from the patient (P), her mother (M), her father (F), and controls (C1–C7). (A) Ethidium bromide stained gel (L = 1 kb ladder). (B) Southern blots hybridised with mutant (ASO-A) and wild type (ASO-C) oligonucleotides.

believed to regulate fibril diameters with the large NH_2-terminal domain of the α1(V) chain projecting out through the gaps between adjacent molecules.[33] If this domain is not properly processed, as suggested by the dextran sulphate labelling experiments in this case, it could have an adverse effect on fibre formation. Disrupting the fibrillogenesis of the closely associated major collagen is thus one mechanism by which the pathological effects of mutations in the minor collagens could be amplified. The electron micrographs of the collagen fibres in the skin clearly suggest this has occurred in this patient.

There have been only a few previous reports of mutations in minor collagens associated with clinical phenotypes. In one, an engineered exon skip in the Col5a2 gene that prohibited removal of the N-propeptide showed abnormal collagen fibrils, spinal deformity, and fragile skin in homozygous transgenic mice.[34] Although mice heterozygous for this Col5a2 mutation did not show an obvious phenotype this may have been because of the nature of the mutation or difficulty in recognising the phenotype in mice. It is not unreasonable to presume that heterozygosity for a COL5A1 mutation would produce a clinical phenotype like other collagen mutations. The stoichiometry of type V collagen chains is such that the COL5A1 product is the dominant partner and mutations in this gene would affect more product and thus be more deleterious than mutations in COL5A2. Type XI collagen is another minor collagen in cartilage. It is homologous to type V collagen and thought to have a similar role in the regulation of type II collagen fibre diameters. Recently, a heterozygous exon skip and a homozygous arginine for glycine substitution in one of its components, COL11A2, were associated with forms of Stickler's syndrome.[35] Furthermore, a recessive mutation of another component of type XI collagen, Col11a1, was identified as the cause of the lethal chondrodysplasia in Cho/Cho mice.[36]

We believe these observations support the conclusion that the heterozygous COL5A1 mutation identified here causes the ultrastructural dysmorphology and clinical phenotype in this sporadic Ehlers-Danlos syndrome patient and provides the first report of a structural abnormality of this protein in humans.

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