A T$^+6$ to C$^+6$ mutation in the donor splice site of COL3A1 IVS7 causes exon skipping and results in Ehlers-Danlos syndrome type IV

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Abstract

Ehlers-Danlos syndrome type IV is usually caused by mutations in COL3A1, the gene coding for type III collagen. In a woman with a milder form of this disease, analysis of type III collagen synthesised by her cultured skin fibroblasts showed an apparently shorter form of the protein. Amplification of overlapping cDNAs, encoding the triple helical region of the molecule, showed a deletion near the 5' end of the gene. Sequencing showed that exon 7 was missing from the cDNA sequence.

Analysis of genomic DNA showed that this was the result of a T$^+4$ to C$^+4$ mutation in the donor splice site of intron 7. The proband's parents and 35 normal controls were homozygous for T$^+4$ at this position, indicating that the C$^+4$ mutation was causative. (J Med Genet 1993;30:376–80)

Of the various types of Ehlers-Danlos syndrome, the most severe is type IV (EDS IV). 1 Patients with this disease can present with a variety of clinical signs. These range from the severe acrogeric type with generalised thin skin, a pinched nose, thin lips, lobeless ears, and premature ageing, to a relatively mild phenotype with variably thin skin, joint laxity, and subtle facial features reminiscent of the more severe phenotype. Fragile blood vessels and hollow organs make EDS IV particularly life threatening, in both the severe and mild forms alike.

Early studies showed that fibroblasts from EDS IV patients secreted lower than normal amounts of type III procollagen. 2 Later, the disease was linked to COL3A1, the gene encoding this protein. 3 More recently, with the publication of full length cDNA sequence and partial characterisation of the gene structure, detailed analysis of mutations in EDS IV patients has become possible. Defects have included point mutations, 4–7 large and small deletions, 8–10 and faulty processing of pre-mRNA. 11–14 Most exon skipping mutations have had G$^+1$ to A substitutions in donor splice sites, causing severe EDS IV. Exceptions are an IVS25 G$^+5$ to T substitution which caused severe EDS IV, 14 and an IVS30 G$^+1$ to A mutation, which resulted in phenotypic overlap between EDS IV and familial aortic aneurysms. 15

Here we have characterised a new exon skipping mutation of COL3A1. Despite only mild external signs of EDS IV the patient had several dissecting aneurysms. A substitution of IVS7 T$^+6$ led to skipping of exon 7. This is the most 5', completely triple helix encoding exon, of type III collagen, since exon 6 codes partially for the N-peptidase cleavage site and the first nine amino acids of the triple helix.

Materials and methods

THE PATIENT

A 32 year old woman presented with severe right sided abdominal pain suggestive of renal colic. Ultrasound examination showed an infarcted right kidney. Later she developed intermittent claudication of the left leg which improved and eventually disappeared. Angiography showed a right sided renal artery occlusion with an aberrantly supplied lower pole. The left iliac artery was stenosed with a possible dissection. Earlier, while still a student, she developed a transient hemiparesis which was attributed to migraine but with a slightly abnormal arch angiogram. Four years before her renal problems she perforated her bowel shortly after the delivery of her only child by caesarean section. She had varicose veins since teenage which, although surgically treated three times, had relapsed. Her parents, a sister, and son were clinically normal.

On clinical examination she had large eyes, lobeless ears, slightly thin skin over the dorsum of the hands with flattened finger pulps. There was capillary prominence over the shoulders and sacrum, loose jointedness of the knees and wrists, and minor varicose veins. She was classified as having mild vascular non-acrogeric EDS IV.

PROTEIN ANALYSIS

Fibroblast cell cultures were established, grown, labelled, and the procollagens and collagens analysed as previously described. 15

cDNA SYNTHESIS AND AMPLIFICATION

Total cytoplasmic RNA was isolated from cultured skin fibroblasts by lysing with Nonidet P-40 in the presence of vanadyl ribonucleoside complex. 16 First strand cDNA synthesis was performed as described previously 4 using one of five antisense primers (see below). Synthesised cDNAs were then amplified as previously described, 4 by the polymerase chain reaction, 17 using the appropriate pair of primers. Those products for which sequencing was required were blunt end ligated into

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| CB peptides | 3 7 6 1 8 2 4 5 9 |
| cDNA | 635 | 815 4105 | 55 | 95 | 633 813 | 4103 | 53 | 93 |
| PCR products | 759 bp | 654 bp | 480 bp | 786 bp | 771 bp |

Figure 1 Schematic representation of type III collagen cDNA and the position of oligonucleotides. The helical region of type III collagen is represented by the upper line. The position of the cyanogen bromide peptides is indicated, except CB10, which is situated between CB8 and CB2. The site of each oligonucleotide is indicated by the arrows which also show their direction 5' to 3'. The size of five overlapping cDNA PCR products are shown as bars below the cDNA.

M13mp18 after treatment with polynucleotide kinase to phosphorylate their 5' termini.

OLIGONUCLEOTIDE PRIMERS USED FOR cDNA AMPLIFICATION

The primers CB4103 and CB4105 were used to amplify cDNA encoding peptides CB4, CB2, and CB10. Similarly, the primers CB813 and CB815 were used to amplify cDNA for peptides CB8 and CB1, while primers CB633 and CB635 amplified CB6, CB7, and CB3. The first nine base pairs of each oligonucleotide code for an EcoRI restriction enzyme site.

CB4103 5'GGGGAATTCAGAGACTTCC-AAGACCTCTCCTTTCA3' antisense
CB4105 5'GGGGAATTCAGAGACTTCC-GTCCCTGAGGTCA3' sense
CB813 5'GGGGAATTCAGAGACTTCC-GGGATGCCCCTCAT3' antisense
CB815 5'GGGGAATTCAGAGACTTCC-CCTGGTGAGTAAGGC3' sense
CB633 5'GGGGAATTCAGAGACTTCCAGGAAT GCCAGCGGACCAT3' antisense
CB635 5'GGGGAATTCAGAGACTTCCAGGAAT GCCAGCGGACCAT3' sense

Primers used to amplify cDNA encoding CB9 and CB5 were as previously described.6,10 Their positions are shown in fig 1.

GENOMIC DNA AMPLIFICATION

Two different genomic DNA products, containing either the whole 54 bp exon 7 or part of it, were amplified for sequencing. The larger (~1560 bp) product was synthesised using primers within exon 6 (CB635, see above) and exon 8 (E8, see below). This was incubated with the restriction enzyme Rsa1 and the fragments blunt end ligated into M13mp18 before sequencing.

A smaller genomic DNA fragment (~560 bp) was amplified using the primers CB633 E7 5'GGTACATCTGTTACATGCTTTACC-CCAGGGATGAGTC3' sense and E8 5'GGGGTACTGTTATCCCCAGAGGA3' antisense. These amplified the 3' end of exon 7, intron 7, and the 5' end of exon 8. This was also blunt end ligated into M13mp18.

Amplification of these DNA fragments was achieved through 10 cycles consisting of 95°C for 1.5 minutes, 60°C for 1.5 minutes, and 72°C for four minutes followed by an additional 30 cycles consisting of 95°C for one minute, 60°C for one minute, and 72°C for three minutes.

SEQUENCING

Individual subclones were sequenced using Sequenase (United States Biochemicals Corp) as described by the manufacturer.

ALLELE SPECIFIC OLIGONUCLEOTIDE (ASO) HYBRIDISATIONS

Amplified genomic DNA from the proband, various members of her family, and normal controls were slot blotted in duplicate onto nitrocellulose and hybridised to ASOs coding for either IVS7 T* 5'GGTTCCCCCTGT-AAGT3' or IVS7 C* 5'GGTTCCCCCTGT- TAAGC3'. Hybridisation and washing conditions were essentially as described by Wood et al.18

Briefly, prehybridisation was at 37°C for 16
hours in 0.9 mol/l NaCl/0.09 mol/l Na citrate (6 × SSC), 50 mmol/l sodium phosphate pH 6.8, 5 × Denhardt’s solution (1 mg/ml each of albumin, polyvinylpyrrolidone, Ficoll), and yeast tRNA at 0.1 mg/ml. Hybridisation was in the same solution containing a labelled ASO. After hybridisation the filters were rinsed three times in 6 × SSC at 4°C and then washed twice for 30 minutes in 6 × SSC at 4°C. The filters were then rinsed twice with 3 mol/l Me₃NCl, 50 mmol/l Tris-HCl pH 8.0, 2 mmol/l EDTA, 0.1% SDS at 37°C, followed by two washes in the same solution at 47–48°C for 20 minutes. At each stage the filters were washed separately.

Results and discussion

Initial analysis of the procollagens synthesised by the patient’s cultured skin fibroblasts showed poor secretion of type III procollagen (fig 2A). When the pepsin treated collagens were examined, an extra protein band was visible in the patient’s samples. It was retained intracellularly and migrated slightly faster in SDS-polyacrylamide gel electrophoresis than normal α1(III) chains, giving the appearance of a broad band (fig 2B). These results suggested synthesis of a shortened form of type III collagen which was very poorly secreted. To test this hypothesis cDNA encoding the entire triple helical region of type III collagen was amplified to detect possible deletions.

The cDNA was amplified as five overlapping products (fig 1). These had originally been designed to encode complete cyanogen bromide peptides, for use in combination with peptide mapping of mutations.6710 This time localisation of the mutation was possible without previous peptide mapping (fig 3). Whereas four of the primer pairs produced amplified products identical to control samples, the amplification product encoding peptides CB6, CB7, and CB3 produced two bands. These were therefore cloned into M13 and sequenced. Several subclones lacked the 54 bp exon 7 (fig 4) suggesting that the mutation caused skipping of this exon during RNA splicing.

Genomic DNA (exons 6 to 8 of COL3A1 -1560 bp) from the proband was amplified and cut with RsaI, for which at least one site occurred (within exon 7). The products were cloned into M13 and sequenced to analyse the donor/acceptor splice sites flanking exon 7. Because of a bias in the type of subclones obtained (only two produced the intron 7 donor splice sequence), a second amplification using primers within exon 7 and exon 8 was performed. This product (~560 bp) was also cloned and sequenced to determine the IVS7 donor splice sequence which was under-represented in subclones of the larger PCR product.

Sequencing showed that the donor/acceptor splice sites of the patient’s two alleles differed only at IVS7*6 which was either a T or C.

Figure 5 Splice site sequences adjacent to exon 7. Exon sequences are shown in bold capitals, intron sequences are in lower case. Donor and acceptor splice sites and possible lariat branch points are shown in bold type. IVS7*6 is either T or C.
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![ASO hybridisations. Amplified DNA from the patient (A1), her mother (B1), father (C1), son (A2), and sister (B2), and normal controls (G2, A3, B3, C3) were slot blotted onto nitrocellulose and hybridised to either IVS7 C\(^{\ast}\) or IVS7 T\(^{\ast}\) as indicated.](image)

Figure 6

residue (figs 4 and 5). Because this position is redundant in the consensus vertebrate 5' donor splice sequence, immediate members of the proband's family and 35 controls were tested with ASOs. The region was amplified, slot blotted, and hybridised to ASOs coding for either IVS7 T\(^{\ast}\) or IVS7 C\(^{\ast}\). After washing with tetramethylammonium chloride the filters were autoradiographed (fig 6). Whereas IVS7 T\(^{\ast}\) hybridised to all samples, IVS7 C\(^{\ast}\) annealed only to DNA from the proband. Thirty-five unrelated control samples (data are only shown for four), and more notably her unaffected parents, did not possess the C\(^{\ast}\) sequence, neither did her clinically normal son and sister.

This IVS7 T\(^{\ast}\) to C\(^{\ast}\) transversion of COL3A1, like the more common G\(^{\ast}\) mutations previously described in this gene,\(^{11,12}\) alters the donor splice site consensus sequence A-2G-1/G+3T-2A.\(^{13,14}\) This causes the preceding exon to be skipped. The +6 position can be redundant in the donor splice site; however, when the -1 and -2 positions are not AG then the +4, +5, and +6 positions tend to conform to the consensus.\(^{21}\) In the case of exon 7 of COL3A1 the last two nucleotides are CT and so presumably a T\(^{\ast}\) becomes essential for recognition by U1RNA, which binds to the donor site during normal splicing.\(^{22}\) Similar observations have been made for IVS1 T\(^{\ast}\) of the \(\beta\) globin gene,\(^{23}\) IVS25 G\(^{\ast}\) of COL3A1,\(^{14}\) IVS14 G\(^{\ast}\) of COL1A1,\(^{25}\) and IVS33 G\(^{\ast}\) of COL1A2.\(^{25}\) These cause \(\beta\)-thalassaemia, EDS IV, and osteogenesis imperfecta (COL1A1 and COL1A2) respectively.

Mutations of G\(^{\ast}\) to A in various introns of COL3A1 can produce quite different splicing patterns. Either high levels of exon skipping or insertion of in frame intron sequences are produced by different mutations.\(^{12}\) Experiments to determine the splicing pattern from the IVS7 C\(^{\ast}\) and other exon skipping mutations in COL3A1 are in progress. Most of the shortened form of type III collagen, produced by the proband's fibroblasts, appears to be retained within the cell layer. This is in contrast to a nine amino acid deletion which is efficiently secreted and causes a more severe clinical phenotype.\(^{10}\) In vivo the N-terminal position of this deletion may also contribute to the mild phenotype as most of the helix C-terminal to this exon will fold normally, from the C- to the N-terminal end. This is in contrast to C-terminal deletions which cause overmodification of the helix N-terminal to the site of mutation.\(^{13}\)

In summary, unlike most exon skipping mutations of COL3A1, this patient does not have the acrogeric form of EDS IV, but more closely resembles the IVS20 G\(^{\ast}\) to A mutation which presented with phenotypic overlap between EDS IV and aortic aneurysms. Why these two mutations produce a milder phenotype is unclear but it is likely to be complex and involve patterns of missplicing, secretion of abnormal protein from the cell, and position within the collagen helix.

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12. Kuivaniemi H, Kontusaari S, Tromp G, Zhao M, Sabel C, Prockop DJ. Identical G\(^{\ast}\) to A mutations in three different introns of the type III procollagen gene (COL3A1) produce different patterns of RNA splicing in
A T+6 to C+6 mutation in the donor splice site of COL3A1 IVS7 causes exon skipping and results in Ehlers-Danlos syndrome type IV.

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