ONLINE MUTATION REPORT

Met>Val substitution in a highly conserved region of the pro-α1(I) collagen C-propeptide domain causes alternative splicing and a mild EDS/OI phenotype

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Type I procollagen is a fibrillar collagen consisting of two pro-α1(I) chains and one pro-α2(I) chain. This procollagen heterotrimer contains a central triple helical domain that is flanked by amino- and carboxy-terminal propeptides (N- and C-propeptides), which are cleaved off after assembly and secretion. The C-propeptides, involved in the formation of post-translational disulphide bonds, are responsible for the correct alignment of the three individual procollagen chains and direct chain–chain recognition.

Mutations in the genes coding for type I procollagen (COL1A1 and COL1A2) have been found in osteogenesis imperfecta (OI) and in the arthrochalasis type of Ehlers-Danlos syndrome (previously EDS types VIIA and B). The heritable brittle bone disease osteogenesis imperfecta has a broad clinical spectrum, ranging from mild to lethal forms.[1] The mildest form, type I OI, is usually caused by mutations generating a premature termination codon and leading to COL1A1 haploinsufficiency. All the procollagen type I that is formed is structurally normal, but its amount is decreased. The more severe forms, types III and IV OI, and the lethal type II OI are generally caused by mutations affecting the structure of type I procollagen. The most common mutations are missense mutations which substitute a crucial glycine residue, occurring in every third position of the triple helix, for a bulkier amino acid. The other mutations are in-frame single exon deletions, splicing defects, and small in-frame insertions and deletions.

In addition to the more than 200 type I procollagen mutations identified in OI, a limited number of mutations in these genes have been reported in EDS. The arthrochalasis type of EDS is caused by mutations leading to the skipping of exon 6 in either the pro-α1 or the pro-α2 chain of procollagen type I.[2] Missplicing or skipping of this exon results in the removal of the cleavage site for the N-proteinase, leaving the N-propeptide of the mutant chains attached to the mature collagen type I.

A rare class of mutations are those located in the C-propeptide of type I procollagen. Procollagen assembly is initiated by folding of the C-propeptides and the formation of intra- and interchain disulphide bonds allowing nucleation of the collagen triple helical domain. Mutations in this C-propeptide domain can impair C-propeptide folding and can delay chain association. Only 13 mutations in the C-telopeptide and the C-propeptide of the pro-α1(I) collagen chain and two mutations in the C-propeptide of the pro-α2(I) collagen chain have been reported so far. Of these C-propeptide mutations, nine have been associated with severe or lethal OI,[3–7] and six with milder OI phenotypes.[8–14] Those resulting in severe or lethal OI phenotypes alter the structure of the C-propeptide but do not prohibit the incorporation of mutant collagen chains into the collagen type I heterotrimers. As such, these mutations interfere with overall collagen fibrillogenesis and post-translational modifications, leading to the formation of overmodified and structurally abnormal type I procollagen.[3–7] The few mutations which have been associated with a milder OI phenotype lead to the production of structurally normal type I procollagen, although in reduced amounts, because the mutant chains

Key points

- Mutations in the triple helical domain and in the N-propeptide domain of the pro-α1(I) and the pro-α2(I) collagen chains have been identified in osteogenesis imperfecta (OI) and in the arthrochalasis type of Ehlers-Danlos syndrome (EDS), respectively. A rare class of type I procollagen mutations are those located in the region coding for the C-propeptide domain of pro-α1(I) collagen, which generally lead to a severe or lethal OI phenotype.
- We have studied skin fibroblasts from a patient presenting a very mild EDS/OI phenotype. SDS-PAGE gels showed a reduction, but no structural abnormalities, of type I (pro)collagen. A missense mutation (3790A>G) in a conserved region of the pro-α1(I) collagen C-propeptide domain was identified, which caused alternative splicing and generated two different mutant transcripts. The first mutant transcript harboured an M1264V substitution and the second carried an out-of-frame deletion leading to the introduction of a premature termination codon (PTC) in exon 50.
- Based on the biochemical findings and the secondary structure prediction program, we postulate that, even if the first mutant transcript is translated, it does not disturb normal collagen synthesis. The second mutant transcript is predicted to generate a truncated pro-α1(I) collagen protein, lacking seven of the eight critical cysteine residues in the C-propeptide, which cannot participate in collagen type I trimerisation (functional haploinsufficiency).
- This is the first C-propeptide mutation in the pro-α1(I) collagen chain leading to a very mild EDS/OI phenotype, and as such expanding the spectrum of phenotypes associated with C-terminal mutations in procollagen type I.

Abbreviations: CSGE, conformation sensitive gel electrophoresis; EDS, Ehlers-Danlos syndrome; NMD, nonsense mediated mRNA decay; OI, osteogenesis imperfecta; PTC, premature termination codon; SAP, shrimp alkaline phosphatase

are not incorporated into the type I procollagen heterotrimers (haploinsufficiency).[8–14]

We report here a novel missense mutation in a conserved region of the C-propeptide of the pro-α1(I) collagen chain leading to a very mild EDS/OI-like phenotype. The mutation results in the substitution of a methionine residue by a valine and, at the same time, activation of a new cryptic splice donor site.

METHODS

Clinical summary

The patient, a 10 year old girl, is the oldest child of a healthy non-consanguineous couple. She has a younger sister without any medical problems. The delivery of the proband was complicated by fracture of the clavicula and a pneumothorax. Postnatally, no further fractures occurred. Although her gross motor development was retarded, her intellectual and fine motor development were normal. She walked alone at the age of 21 months. At the age of 4 years an adenoidectomy was performed because of obstructive sleep apnea syndrome. An audiogram and an echocardiography were normal and a skeletal survey did not show any deformities. *x* Ray analysis showed reduced mineralisation of the cortex of the skull and carpal bones. There were no Wormian bones present on skull *x* ray. DXA scanning results were not available. At the age of 7 years 4 months her height was 133.5 cm (97th percentile), weight was 25 kg (between 50th and 75th percentile), and head circumference 54.1 cm (95th percentile). Arm span was 131 cm and BMI was 14 kg/m^2 (10th percentile). Teeth were of normal size, shape, and colour. No caries were present. The child had a long narrow facies with a high arched palate and malpositioning of the teeth. She was treated with an orthodontic apparatus for teeth malpositioning from the age of 6 years. She had a Beighton score of 8/9 at the age of 7 years. To date, she has mild blue sclerae, mild hyperelasticity of the skin, genu recurvatum, and mild joint hypermobility mainly at the hands and wrists. No large atrophic scars, striae distensiae, large haematomas, or skin ''fat herniations'' were present. Only a small scar on the thorax was noticed as a result of a thoracal drain because of the neonatal pneumothorax.

Biochemical collagen analysis

Fibroblast cultures were established from a skin biopsy taken from the proband and maintained under standard conditions.

At confluence, cells were labelled with ^{14}C-proline. After 20 h, the medium was removed and supplemented with proteinase inhibitors (0.1 mg PMSF, 0.1 mg NEM, and 2 mM EDTA pH 7.5). The cell layer was trypsinised, and the cells were collected by centrifugation and were lysed in 0.5% Triton X-100 in 0.5 M acetic acid. The supernatant was used for collagen analysis.

Procollagen samples were isolated from the medium by alcohol precipitation. Pepsin digestion was performed to convert the procollagen molecules to collagen molecules. (Pro)collagen separation was analysed by SDS-PAGE and visualised by autoradiography. Two-dimensional peptide mapping was performed by in situ digestion with cyanogen bromide (CNBr) followed by electrophoresis in the second dimension.[15] The thermal stability of collagens was determined as described previously.[16]

COL1A1 null-allele testing

Total RNA was isolated from cultured skin fibroblasts by Trizol (Life Technologies) and treated with RNase-free DNase (Invitrogen). For the conversion to cDNA, Moloney murine leukemia virus reverse transcriptase was used in combination with random hexanucleotide primers (Invitrogen).

Genomic DNA was isolated from the fibroblast cultures using standard procedures (Qiagen).

The COL1A1 null-allele assay relies on the detection at the genomic and the cDNA level of two polymorphisms: a MnlI restriction site[17] and a 4 bp insertion[18] located in the 3′UTR of the COL1A1 gene. The fragment with the 4 bp insertion was amplified with PCR and analysed by fragment analysis on the ABI 3100 (Applied Biosystems).[19] The fragment carrying the MnlI polymorphism was analysed with the primer extension technology (SNaPshot, Applied Biosystems). For this purpose, the fragment was amplified in a PCR reaction and purified by addition of shrimp alkaline phosphatase (SAP). The primer extension reaction (96℃ for 10 s, 50℃ for 5 s, 60℃ for 30 s, 25 cycles) was performed using the following primer: 5′-GAC AAG CAA CCC AAA CTG AAC CCC C-3′. After primer extension, the reaction was purified by addition of SAP. Analysis was performed using the ABI 3100 GeneScan (Applied Biosystems).

Detection of the different COL1A1 alleles

We used the primer extension technology as described above. A primer corresponding to the sequence 5′ to the mutation (primer 1, P1) was designed: 5′-CGC CCG CAC CTG CCG TGA CCT CAA G-3′. The primer extension reaction was performed at the gDNA and the cDNA of the patient and a control sample, and the corresponding peak area ratio was measured. From this, the level of expression of the different alleles (WT and mutant) was estimated. In order to determine the effect of the mutation on the expression of the mutant and the normal alleles, a primer was designed the last nucleotide from which corresponds to the mutated nucleotide (primer 2, P2): 5′-CGC CCG CAC CTG CCG TGA CCT CAA GG-3′. The cDNA of the patient and a control sample were analysed as described above. Primer extension results in the addition of the nucleotide immediately adjacent to the mutation, allowing us to distinguish between the different transcripts.

Mutation analysis

PCR was performed on cDNA, using primers amplifying the COL1A1 and the COL1A2 coding region and the amplimers were analysed by conformation sensitive gel electrophoresis (CSGE) as described.[20]

Fragments showing an abnormal migration shift were cloned using the TOPO cloning kit (Invitrogen). Positive clones were selected and sequenced, using the ABI 3100 Automatic Sequencer (Applied Biosystems).

The pathogenicity of the mutation was confirmed by sequence analysis at the gDNA level in both parents and in 50 control samples.

The nucleotide position of the mutation is reported with respect to a reference cDNA sequence with accession number Z74615 and according to the new guidelines of the Human Collagen Database (nucleotides numbered from the first base of the start codon).

RESULTS

Collagen protein analysis

In the patient, the intensity of the bands corresponding to (pro)collagen type I was reduced when compared to a control sample. This reduction was only visible in the gels representing the medium collagen fraction (fig 1A). Overexposure of the gels representing the cell layer collagen fraction did not reveal any extra bands, thus excluding the possibility that abnormal collagen type I is produced (10 day exposure; fig 1B). Two-dimensional CNBr digestion of the α1(I) and the α2(I) collagen chain did not reveal any abnormal shifts (data not shown). Thermal stability of the collagen type I chains in the patient was comparable to that of a control sample (data not shown).

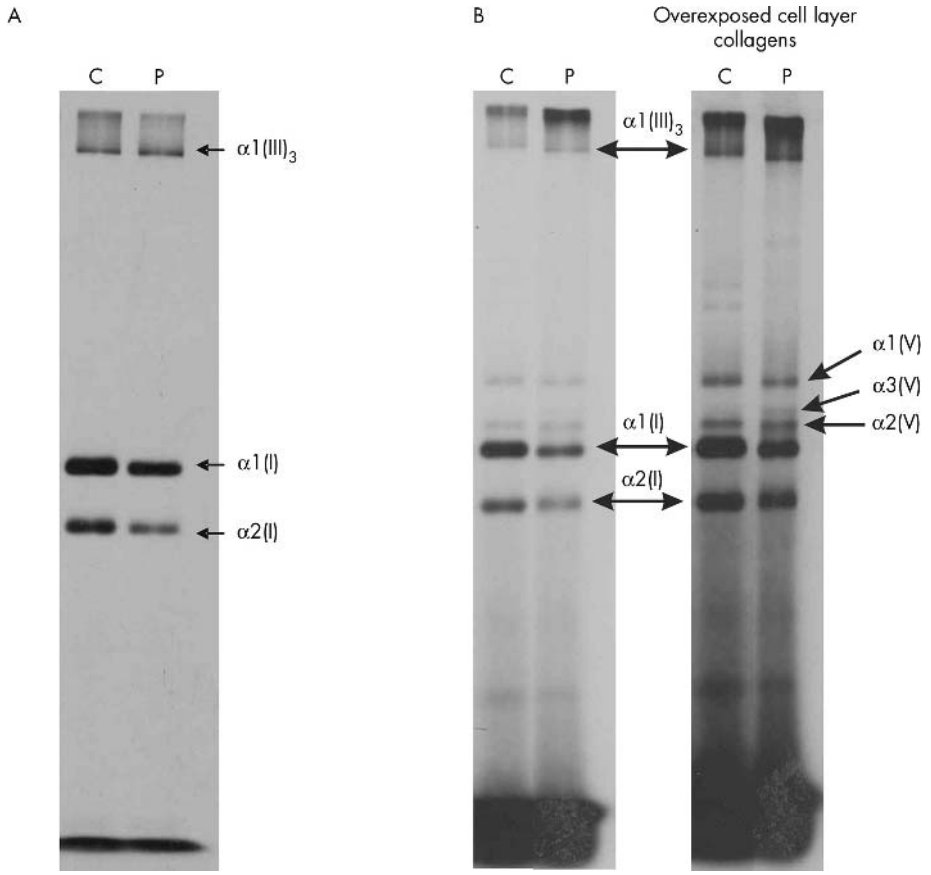

Figure 1 Electrophoresis of pepsin-digested fibroblast collagens. (A) Collagens secreted in the medium showed a marked reduction in the intensity of the collagen type I molecules when compared to a control sample. Fibroblasts of the patient (P) and an unrelated control (C) were examined. The arrows indicate the migration positions of type I collagen α1(I) and α2(I) chains and type III collagen ($\alpha1(III)_3$). (B) Cell layer collagens from the patient showed a normal appearance and intensity of the collagen type I bands when compared to a control sample (1 day exposure). Overexposure of the cell layer collagens did not show any alteration of the collagen type I molecules (10 day exposure).

COL1A1 null-allele detection

To test for the presence of a non-functional COL1A1 allele, we performed a COL1A1 null-allele detection assay. However, the patient was homozygous for both polymorphisms (data not shown), so the possibility of a COL1A1 null-allele could not be excluded in this way.

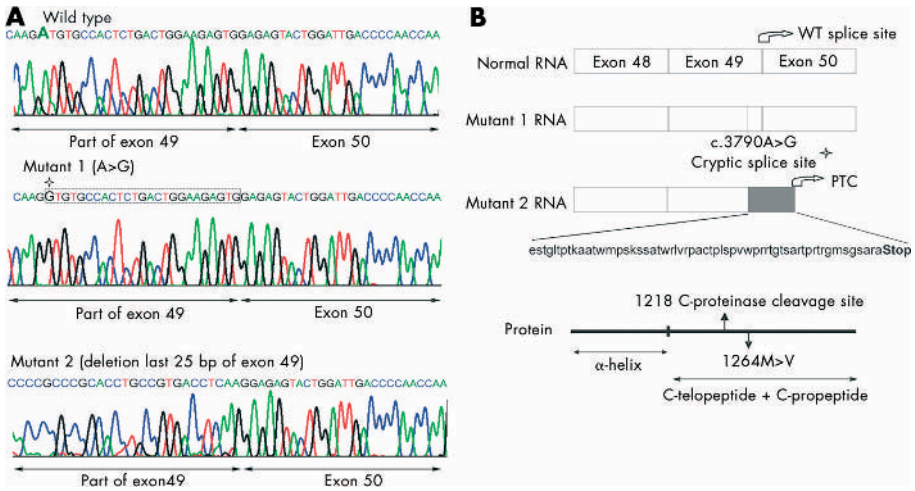

Figure 2 Sequences of the three different mRNA transcripts. (A) The first sequence shows part of the normal sequence of the COL1A1 exon 49 and exon 50. The middle sequence is the first mutant transcript (mutant 1) harbouring the 3790A>G missense mutation, resulting in the M1264V substitution. The bottom sequence is the second mutant transcript (mutant 2) in which the last 25 bp of exon 49 are deleted (marked in a dotted box in mutant 1), resulting in the generation of a PTC in exon 50. (B) Mechanism by which the 3790A>G missense mutation generates two different aberrant transcripts. The mRNA and the protein level are shown. The M1264V mutation is located close to the C-proteinase cleavage site.

Detection of the collagen mutation in the α1(I) mRNA

We analysed the entire mRNA, encoding the pro-α1(I) and the pro-α2(I) collagen chains, using the conformation sensitive gel electrophoresis (CSGE) assay. Screening of the COL1A2 cDNA sequence did not reveal any abnormal shift. In contrast, the fragment coding for exons 48–50 of the COL1A1 gene yielded extra bands (data not shown). Cloning and sequencing of this fragment revealed the presence of two aberrant mRNA transcripts in addition to the normal transcript (fig 2A).

Some clones carried a mutant transcript (mutant 1) with a base transition 3790A>G in exon 49 leading to the substitution of a methionine by a valine at position 1264 of the C-propeptide of the pro-α1(I) collagen chain. Using the Gibrat (GOR3) secondary structure prediction method[21], we showed that this mutation caused only minor alterations in the secondary structure of the C-propeptide domain (data not shown).

Computational analysis of this sequence (mutant 1), using the splice site prediction by neural network program, revealed that the 3790A>G mutation in exon 49 also generates a new cryptic splice donor site with a strength of 0.96 (fig 2B). Identification of a second mutant transcript (mutant 2) proved that the newly generated cryptic splice donor site in exon 49 is used in combination with the normal splice acceptor site of exon 50. As a consequence, the last 25 bp of exon 49 are removed from the mRNA (fig 2A), resulting in a frameshift and in the formation of a premature termination codon (PTC) in exon 50 (COL1A1 haploinsufficiency). At the protein level, this deletion causes the removal of seven of eight crucial cysteines in the C-propeptide (due to the premature termination of translation) and the production of shortened pro-α1(I) collagen chains which cannot align with the normal pro-α1(I) collagen chains and therefore cannot be incorporated into the type I procollagen molecules (fig 2B). Sequence analysis in both parents and 50 unrelated control samples revealed only the normal sequence (absence of the 3790A>G mutation).

Detection of the different COL1A1 alleles

To investigate the pathogenetic effect of the mutation, we estimated the amount of each mutant allele in the mRNA pool. For this purpose we designed a primer specific for the sequence 5′ of the 3790A>G mutation (primer 1, P1; fig 3A). In fig 3B, the result of the primer extension reaction is shown. The blank peaks correspond to the measurements at the gDNA level and the filled peaks to those at the cDNA level. The measurements of the wild type allele, at the gDNA and cDNA level, are represented by the green peaks (incorporating Adenosine). Both peaks have approximately the same height, indicating that equal amounts of DNA at the gDNA and cDNA level are present. The blue peaks correspond to the measurements for the mutant allele (incorporating Guanosine). The peak corresponding to the cDNA level is decreased, suggesting that the expression of the mutant allele is strongly reduced.

We assumed that the reduction in the amount of the total mutant allele was caused predominantly by elimination through nonsense mediated mRNA decay (NMD) of mutant 2, harbouring the PTC generated after the use of the new cryptic splice site. The remaining fraction of mutant mRNA would then consist of mutant 1 carrying the 3790A>G substitution, which leads to the production of M1264V mutant proα1(I) collagen chains (mutant 1). To confirm this hypothesis we designed a primer (primer 2, P2; fig 3A) that distinguishes between the wild type allele and mutant 1 on the one hand and the aberrantly spliced transcript (mutant 2) on the other by using the primer extension technology. Surprisingly, fig 3C shows that mutant 2 remains partly present in the mRNA fraction and thus that this transcript is not fully degraded by the NMD pathway. The presence of both mutant transcripts is also confirmed by cloning and sequencing of the aberrant COL1A1 cDNA fragment.

DISCUSSION

Mutations affecting the splicing of the N-propeptide domain of the pro-α1(I) and pro-α2(I) collagen chains[2] as well as a

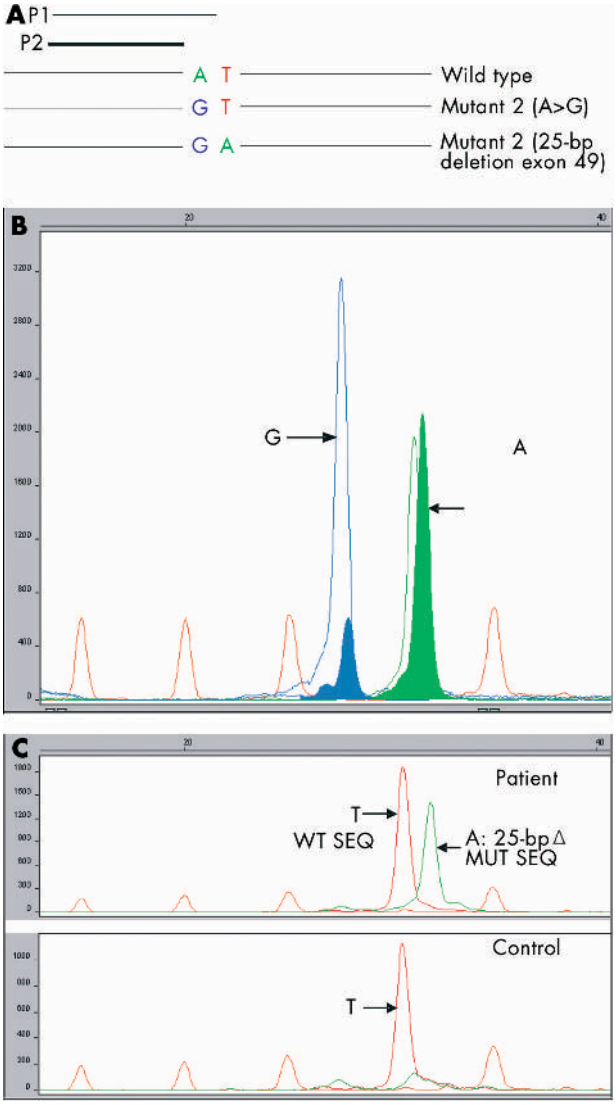

Figure 3 Detection and quantification of allele specific transcript levels of the COL1A1 gene in the patient and in a control sample. (A) Schematic representation of the primer design at the mRNA level for the primer extension technology. P1 is the primer specific for the mutation; this primer will discriminate between the normal (A) and the mutant nucleotide (G). P2 is the primer specific for the nucleotide following the mutation and so we can discriminate between the normal and the mutant 1 transcript and the mutant 2 transcript. (B) Primer extension reaction on the gDNA and the cDNA of the patient with primer P1. This experiment is repeated 5 times and the mean peak area ratio is calculated. Green peaks: adenosine, wild type allele (WT); blue peaks: guanosine, mutant allele. Filled peaks: cDNA measurements; blank peaks: gDNA measurements. (C) Investigation of the presence of the PTC-harbouring allele in the mRNA pool. The primer extension reaction was performed on the cDNA of the patient and on a control sample with primer P2. Green peak: adenosine, red peak: thymine. In the control primer P2 was extended by a thymine, corresponding to the WT+mutant 1. In the patient, two peaks were detected, one corresponding to the WT+mutant 1 and one corresponding to mutant 2.

Table 1 Conservation of the altered methionine residue in this patient

Human α1(I)	SPEGSRKNPARTCRDLK***M***CHSDWKSGEYWI*D*PNQGCNLD
Canis familiaris α1(I)	SPEGSRKNPARTCRDLK***M***CHSDWKSGEYWI*D*PNQGCNLD
Xenopus laevis α1(I)	SPEGTKKNPARTCRDLK***M***CHSDWKSGEYWI*D*PNQGCILD
Trout α1(I)	SPEGTKKNPARTCRDLK***M***CHPDWKSGEYWI*D*PDQGCTQD
Human α2(I)	TPEGSRKNPARTCRDLR***L***SHPEWSSGYYWI*D*PNQGCTMD

Part of the amino acid sequence (single-letter code) of the C-propeptides of four fibrillar pro-α1(I) collagen chains from different species and of the human fibrillar pro-α2(I) collagen chain is aligned. The bold italic letters indicate the M1264V mutation and the italic underlined letters indicate the D1277H mutation described earlier.

few homozygous and heterozygous mutations in the COL1A2 gene[22 23] (personal communication) have been shown to cause OI/EDS overlapping phenotypes. We describe here the first pro-α1(I) C-propeptide mutation associated with a very mild EDS/OI overlapping phenotype. This de novo C-propeptide mutation (3790A>G) causes alternative splicing and generates two different mutant transcripts thought to be responsible for the mild phenotype of this patient. The first mutant transcript harbours an M1264V missense mutation, while the second mutant transcript is generated after the use of a new cryptic splice donor site in exon 49.

The M1264V missense mutation in the first mutant transcript is located in the most conserved region of the C-propeptide of the pro-α1(I) collagen chain (table 1). A previously identified missense mutation (D1277H) in this region leads to lethal OI,[3] changing the most conserved residue in this region from an acidic residue to a basic one. The latter mutation permits chain association but impairs interchain disulphide bonding, slowing down the process of triple helix formation. This results in the formation of stable but structurally abnormal and highly overmodified (pro)collagen type I. This finding was not seen in the patient described here. The (pro)collagen type I chains showed a normal electrophoretic migration pattern and had normal thermal stability, but were reduced in intensity indicating that type I (pro)collagen was reduced. No evidence for structural abnormalities of the (pro)collagen type I chains was found, not even after overexposure of the SDS-PAGE gels. Furthermore, prediction of the secondary structure of the C-propeptide of the M1264V mutant pro-α1(I) collagen chains showed only minor structural alterations. Since no overmodification of (pro)collagen type I was seen on biochemical analysis, we assume that either the M1264V mutant chains are not formed or that, if they are formed, they do not have a major effect on the collagen fibrillogenesis and still allow the formation of structurally normal (pro)-collagen type I.

The second mutant transcript is generated after the use of a new cryptic splice site in exon 49, resulting in an out-of-frame deletion of the last 25 bp of exon 49 and the introduction of a PTC in exon 50. As seen in fig 3B, its expression is strongly reduced, which is consistent with degradation of this transcript by NMD and reduced intensity of (pro)collagen type I observed on the medium protein gels of the patient. We were still able to detect a remnant of this second mutant transcript at the mRNA level, but even after overexposure of the cell layer collagen SDS-PAGE gels we could not detect the presence of a truncated pro-α1(I) collagen chain. Even if this mutant mRNA were translated into protein, the truncated protein would have an altered C-propeptide lacking seven of the eight crucial cysteine residues. As a consequence these mutant pro-α1(I) collagen chains would still not be able to participate in procollagen assembly and would result in a reduction of type I (pro)collagen (functional haploinsufficiency). Although overexposure of the cell layer collagen SDS-PAGE gels could not confirm that this truncated pro-α1(I) collagen chain is formed, the possibility remains that small amounts of the mutant protein are made but rapidly degraded and thus not incorporated into the (pro)collagen type I heterotrimers. Two mechanisms could explain the mild EDS/OI phenotype of this patient. According to a first hypothesis the phenotypic effect of the mutation is predominantly the result of COL1A1 haploinsufficiency caused by rapid degradation of mutant 2, either at the mRNA or at the protein level. Indeed, the fact that the calculated strength of the new cryptic splice donor site is very high suggests that the second mutant transcript is the preferentially generated transcript. COL1A1 haploinsufficiency is usually associated with mild OI, which still covers a broad range of phenotypic severity. In certain instances the skeletal symptoms can be very mild and the soft connective tissue signs can dominate the clinical picture causing an overlap with EDS, as seen in this patient.

Alternatively, the mild phenotype of this patient could result from the combined effect of both mutant transcripts. Based on the biochemical findings, we assume that, if the first mutant transcript is translated, the M1264V mutant chains could participate in procollagen assembly but do not have a harmful effect on the normal function and structure of these molecules. At the same time, M1264V mutant chains could attenuate the haploinsufficiency effect of mutant 2 to some extent and ameliorate the expected OI phenotype of the patient.

In conclusion, we have identified the first C-propeptide mutation in the pro-α1(I) collagen chain leading to a very mild EDS/OI phenotype, thereby expanding the spectrum of phenotypes associated with C-terminal mutations in procollagen type I.

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Electronic database information is as follows: Splice site prediction by neural network: http://www.fruitfly.org/seq_tools/splice.html; Gibrat (GOR3) secondary structure prediction method: http://npsa-pbil.ibcp.fr/cgi-bin/npsa_automat.pl?page=npsa_gib.html.; Database of human type I and type III collagen mutations: http://www.le.ac.uk/genetics/collagen/; NCBI sequence viewer: http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=nucleotide&list_uids=1418927&dopt=GenBank

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