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A homozygous *COL6A2* intron mutation causes in-frame triple-helical deletion and nonsense-mediated mRNA decay in a patient with Ullrich congenital muscular dystrophy

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Abstract Ullrich congenital muscular dystrophy (UCMD) is a severe disorder caused, in most cases, by a deficiency in collagen VI microfibrils. Recessive mutations in two of the three collagen VI genes, *COL6A2* and *COL6A3*, have been identified in eight of the nine UCMD patients reported thus far. A heterozygous *COL6A1* gene deletion, resulting in a mutant protein that exerts a dominant negative effect, has recently been described in a severely affected UCMD patient. Here we describe a patient in whom reverse transcription-PCR analysis of fibroblast RNA suggested a heterozygous in-frame deletion of exon 13 in the triple-helical domain of *COL6A2*, which is predicted to be dominantly acting. However, a homozygous A → G mutation at –10 of intron 12 was found in the genomic DNA. The intron mutation activated numerous cryptic splice acceptor sites, generating normal and exon 13-deleted *COL6A2* mRNA, and multiple aberrant transcripts containing frameshifts that were degraded through a nonsense-mediated decay mechanism. Northern analysis indicated diminished

COL6A2 mRNA expression as the primary pathogenic mechanism in this UCMD patient. Our results underscore the importance of multifaceted analyses in the accurate molecular diagnosis and interpretation of genotype-phenotype correlations of UCMD.

Introduction

Ullrich congenital muscular dystrophy (UCMD, MIM 254090) is a severe disorder affecting both muscle and connective tissue. It is characterized by neonatal muscle weakness, proximal joint contractures, marked distal joint hyperlaxity, and normal intelligence (Bertini and Pepe 2002). Additional clinical features may include kyphosis, scoliosis, torticollis, rigid spine, and hip dislocation. Patients affected with UCMD have delayed motor milestones and may never walk independently. Respiratory function is often severely compromised, leading to respiratory failure requiring noninvasive ventilation from the 1st to 3rd decades of life.

In 2001, UCMD was first shown to result from a deficiency in collagen VI due to recessive mutations in *COL6A2*, one of the three subunit genes encoding the protein (Camacho Vanegas et al. 2001; Higuchi et al. 2001). Since then, mutations in all three collagen VI genes have been reported in nine families, eight of which carry homozygous or compound heterozygous frameshift, nonsense, or splice junction mutations in *COL6A2* or *COL6A3* that result in the absence or severe reduction of collagen VI protein (Demir et al. 2002; Ishikawa et al. 2002). The exception is a severely affected UCMD patient who carries a de novo heterozygous deletion in *COL6A1*, resulting in an in-frame deletion in the $\alpha 1(\text{VI})$ collagen chain that exerts a dominant negative effect (Pan et al. 2003). Hence, UCMD can arise from either recessive or dominant

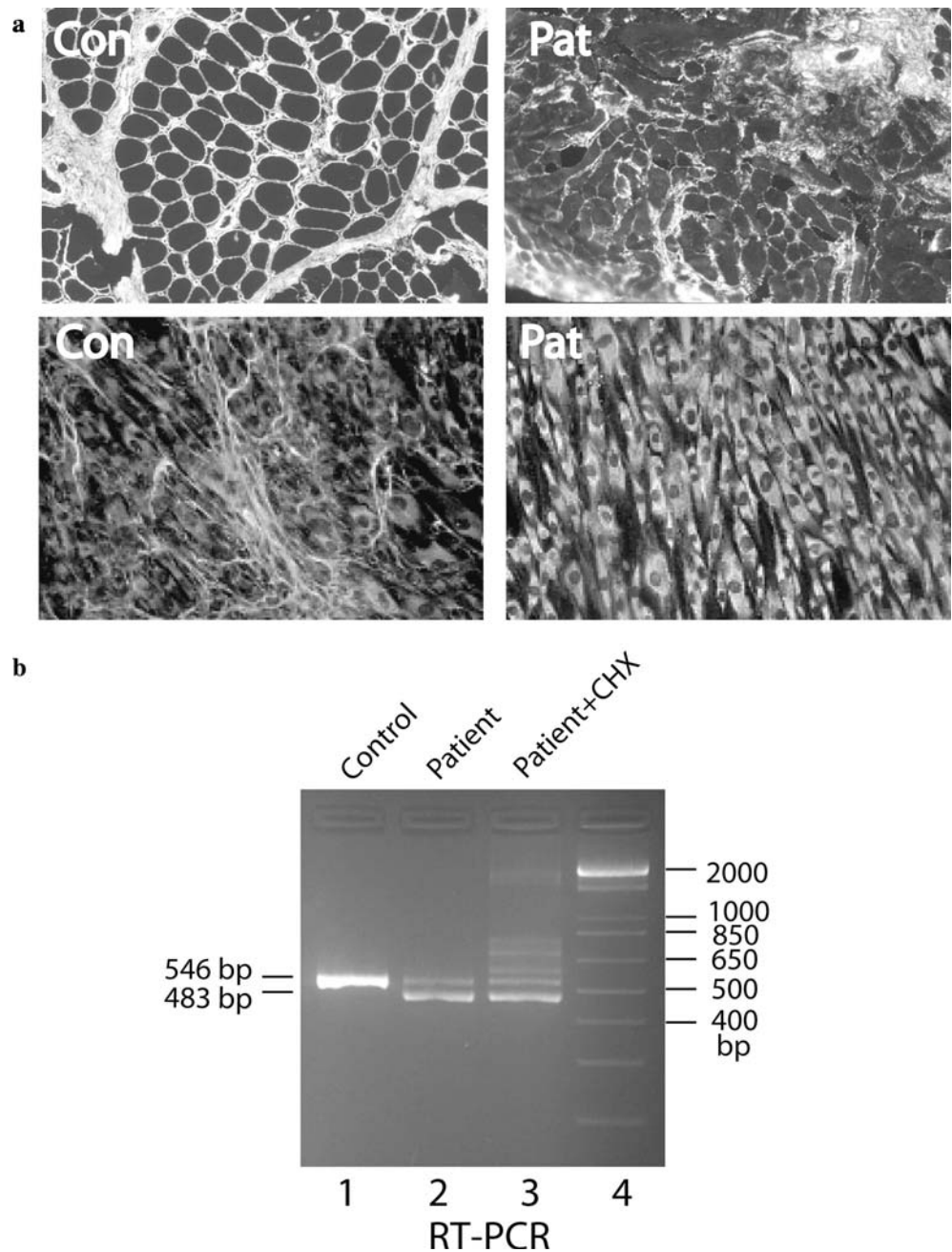
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Fig. 1 a Immunofluorescence analysis of muscle biopsies (*top panels*) and dermal fibroblasts (*bottom panels*) from the control (*Con*) and patient (*Pat*). Muscle biopsies were immunostained with a collagen VI monoclonal antibody (MAB 3303, Chemicon), followed by a biotinylated anti-mouse secondary antibody and streptavidin–Texas red conjugate. Fibroblasts were grown in the presence of 50 $\mu\text{g}/\text{ml}$ L-ascorbic acid phosphate for 4 days post-confluency and stained with an $\alpha 1(\text{VI})$ collagen specific antibody. The antibody reaction was detected with Cy3 conjugated goat anti-rabbit IgG, showing extensive collagen VI microfibrillar network in the control fibroblasts and only cytoplasmic staining in the patient fibroblasts. Original magnifications: $\times 20$. **b** RT-PCR analysis of total RNA from fibroblasts using a primer pair that amplify a 546 bp fragment in the N-terminal portion of the triple-helical domain of the *COL6A2* mRNA. Lane 1 control RNA, lane 2 patient RNA, lane 3 patient RNA from cells treated with 100 $\mu\text{g}/\text{ml}$ cycloheximide, and lane 4 DNA size marker



negative mutations. Previously, dominant mutations in all three collagen VI genes have been associated with the milder Bethlem myopathy, characterized by congenital muscle weakness and multiple joint contractures (Bertini and Pepe 2002).

The $\alpha 1(\text{VI})$, $\alpha 2(\text{VI})$ and $\alpha 3(\text{VI})$ chains of collagen VI are 140, 140 and 260–300 kDa in size, respectively (see Timpl and Chu 1994). Each chain contains a central triple-helical domain of 335–336 amino acids with repeating Gly-Xaa-Yaa amino acid sequences, which is flanked by amino- and carboxyl-globular domains. The three chains are folded, through the triple-helical domains, into collagen VI monomers that are further assembled into dimers and tetramers prior to secretion

from cells. In the extracellular space, the tetramers are associated end-to-end into the characteristic double-beaded collagen VI microfibrils. Thus, mutations affecting the multiple assembly steps can have strong dominant negative effects, resulting in the total absence of collagen VI microfibrils (Pan et al. 2003).

Here, we report a UCMD patient showing an apparently heterozygous in-frame deletion in the triple-helical domain of the $\alpha 2(\text{VI})$ collagen chain by RT-PCR analysis, as a consequence of a homozygous single nucleotide substitution in the intron that causes complex aberrant splicing. This type of mutation mechanism has not been reported previously in UCMD. Our finding is highly relevant to the accurate molecular diagnosis of the disorder.

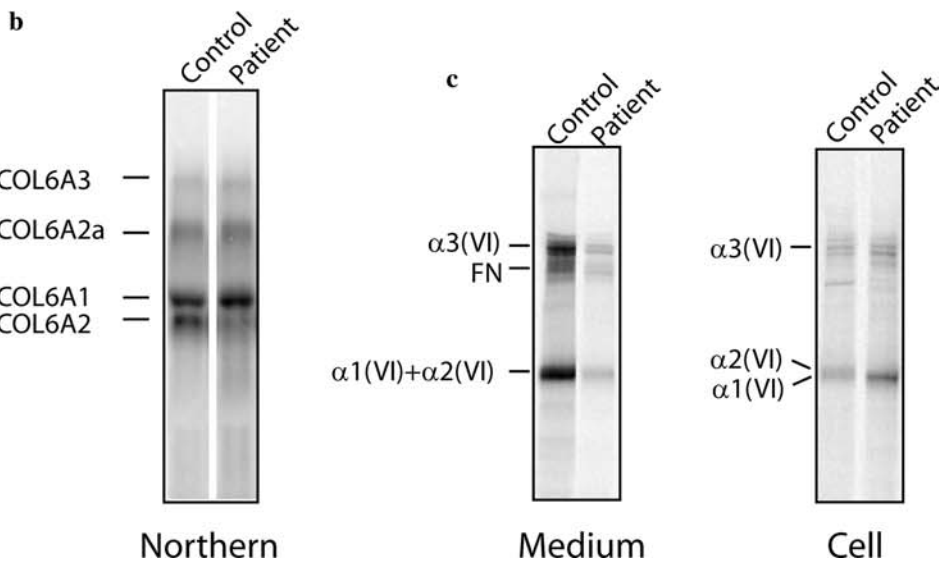
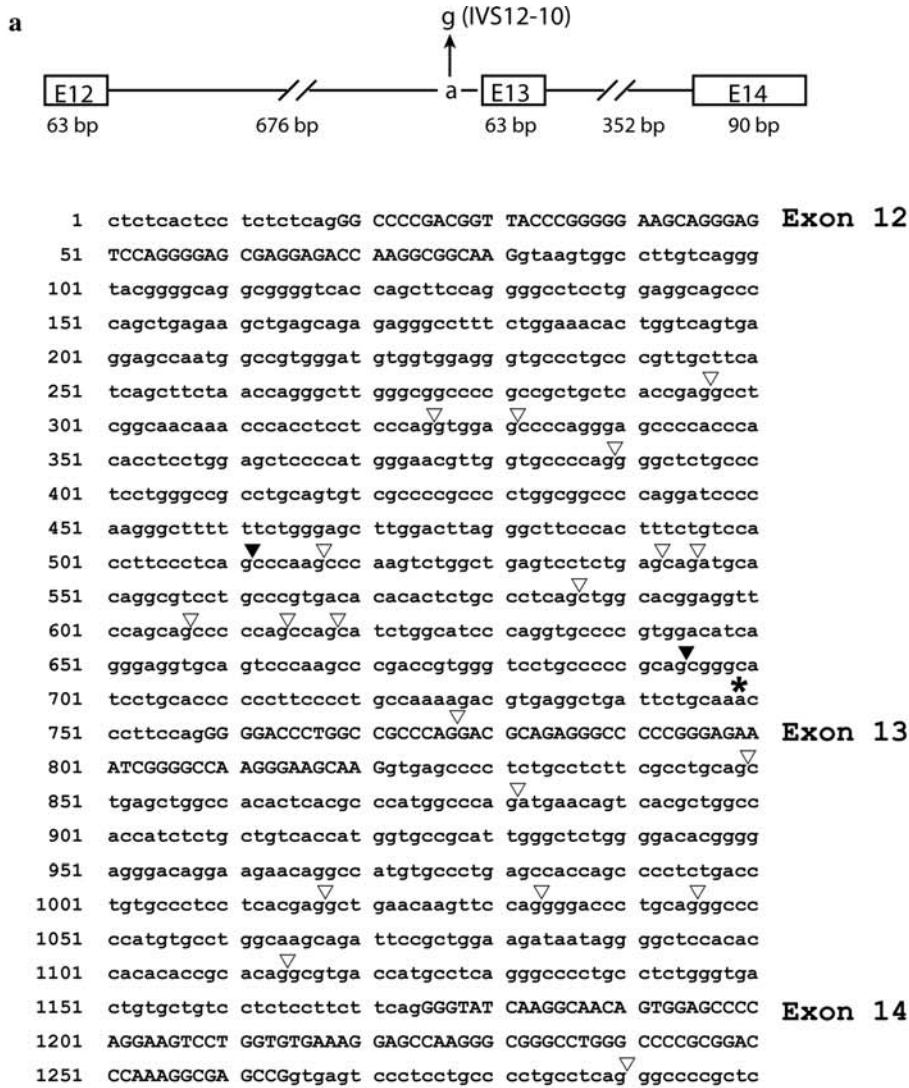




Fig. 2 a *Top*: Schematic diagram of the *COL6A2* genomic region encompassing exons 12–14 (*open boxes*), which encode the triple-helical domain (*top*). A homozygous IVS12-10a → g mutation is present in the genomic DNA of the patient. *Bottom*: Nucleotide sequence of the corresponding region. Exon sequences are in capital letters. Potential splice acceptor sites predicted by computer analysis are depicted by *open arrowheads*, and *filled arrowheads* indicate two sites identified in cDNA clones. *Asterisk* marks the intron 12 mutation in the patient. **b** Northern blot analysis of total RNA (5 µg) from control and patient fibroblasts using a mixture of [³²P]dCTP-labeled cDNA probes for the three collagen VI mRNA. The probes hybridized with the *COL6A1*, *COL6A2* and *COL6A3* mRNA of 4.2, 3.5 and 9–10 kb, respectively, plus an alternatively spliced *COL6A2* mRNA (*COL6A2a*) of 6.0 kb. **c** Immunoprecipitation of culture media (*left*) and cells (*right*) from control and patient fibroblasts with the antibody specific for the α3(VI) collagen chain. Fibroblasts were labeled with [³⁵S]cysteine overnight. Samples were reduced with 25 mM DTT and separated on a 3–8% polyacrylamide gel. Note that a strong band migrated at the position of the α1(VI) collagen chain is seen in the cells from the patient, whereas equal amounts of the α1(VI) and α2(VI) chains, which are not completely resolved in this gel, are observed in the control. In the culture medium, a small amount of fibronectin (*FN*) is also precipitated by the antibody

Materials and methods

Patient

The proband was a 5-year-old Caucasian male. He presented at the age of 3 months with hypotonia, bilateral hip dislocation and recurrent chest infections. His motor milestones were severely delayed: he stood at 31 months but has never achieved independent ambulation. At a recent clinical examination, he showed generalized muscle weakness, affecting more severely the proximal muscle in the limbs and distal laxity involving elbow, fingers, and ankles. Flexion contractures of hips and knees were also present. He had mild spine rigidity.

Muscle biopsy and dermal fibroblasts established from skin biopsy were used in this study. The biological specimen was collected in accordance with the institutional review board-approved human subject protocols.

Mutation detection

Mutation analysis was performed by RT-PCR amplification and direct sequencing of the entire coding regions of all three collagen VI mRNAs using methods and primers described previously (Camacho Vanegas et al. 2001; Pan et al. 2003). The primers used to identify the *COL6A2* mRNA deletion were forward 5'-ATCGGACCTCCTGGCTGCAA and reverse 5'-GGGTCCTGGTAGCTGAATC, which amplified a 546 bp cDNA fragment. Exon 13 plus its flanking intron sequences in *COL6A2* was PCR amplified from the genomic DNA using primers 5'-GACACACTCTGCCCTCAG (forward) and 5'-CTCAAGCTGCAAGGAGGATG (reverse), which amplified a 758 bp fragment. The PCR products were directly sequenced.

Immunohistochemical, molecular and biochemical analyses

Immunohistochemical study of muscle biopsies was performed by incubating 7 µm cryosections with a collagen VI monoclonal antibody (MAB 3303, 1/400 dilution, Chemicon), followed by a biotinylated anti-mouse secondary antibody (Amersham) and streptavidin-Texas red conjugate (molecular probes).

Immunofluorescence, RNA and protein analyses of collagen VI in dermal fibroblasts were carried out as described previously (Pan et al. 2003). Briefly, confluent fibroblasts grown in the presence of L-ascorbic acid phosphate for 4 days were immunolabeled with a polyclonal antibody specific for the α1(VI) collagen chain (Tillet et al. 1994). Northern blot hybridization was performed using total RNA prepared from confluent fibroblasts and [³²P]dCTP-labeled cDNAs encoding the three collagen VI chains as probes. To assess aberrant splicing, fibroblasts were grown in 100 µg/ml cycloheximide (Sigma) overnight prior to total RNA isolation (Bateman et al. 1999). For immunoprecipitation, fibroblasts were labeled overnight with [³⁵S]cysteine, and the resulting cells and culture medium were precipitated with a polyclonal antibody specific for the α3(VI) collagen chain (Specks et al. 1992).

Results

Immunohistochemical examination of the muscle biopsy from the patient showed variation in fiber size, marked reduction of collagen VI in the basal lamina surrounding the fibers, and positive staining in the connective tissue between the fibers and in the perivascular regions (Fig. 1a). Immunofluorescence analysis of the patient fibroblasts showed strong cytoplasmic staining and a complete absence of collagen VI microfibrils in the extracellular matrix. These observations indicated the presence of a collagen VI defect in the patient. Therefore, the coding regions of the three collagen VI mRNAs were RT-PCR amplified with 25 primer pairs in their entirety, and the resulting products were analyzed by agarose gel electrophoresis. A primer pair specific for the triple-helical domain of the *COL6A2* mRNA amplified two cDNA fragments, the expected 546 bp band and a slightly shorter fragment (Fig. 1b, lane 2). DNA sequencing revealed that the shorter fragment contained a 63 bp deletion, corresponding to exon 13 (Fig. 2a). The larger PCR product showed no deviation from the *COL6A2* mRNA reference sequence. The results suggested that the patient might carry a heterozygous splice junction mutation causing exon skipping. A number of single nucleotide changes were also identified by sequencing the entire coding regions of the three chains, three of which result in amino acid substitutions in *COL6A3* (Table 1). These nucleotide changes have frequently been observed in Europeans (Lampe et al. 2005), and

thus are unlikely to be pathogenic. The patient is heterozygous for a 2667G → A polymorphism in *COL6A1*, suggesting that both *COL6A1/COL6A2* alleles are present.

The genomic DNA encompassing exons 12–14 (~1.2 kb) was next PCR amplified and sequenced. Unexpectedly, the only sequence change detected was a homozygous A → G transition at position –10 of intron 12 (Fig. 2a). His unaffected mother is heterozygous for this A → G change. The genomic DNA of his father was not available for analysis.

Close inspection of the RT-PCR products showed that the normal and shortened cDNA fragments were not present in equal quantity, an observation inconsistent with the postulation of a heterozygous splice junction mutation. Northern blot hybridization was performed to examine whether the intron mutation resulted in quantitative or qualitative changes in the *COL6A2* mRNA. As shown in Fig. 2b, the *COL6A2* mRNA level in the patient was substantially reduced compared to the normal control, whereas the amounts of the *COL6A1* and *COL6A3* mRNAs in the patient and control were similar. The data suggested that the majority of the *COL6A2* mRNA in the patient fibroblasts was degraded, most likely through nonsense-mediated mRNA decay (Frischmeyer and Dietz 1999). This possibility was further investigated by RT-PCR analysis of total RNA prepared from the patient fibroblasts that had been treated with cycloheximide to inhibit RNA degradation. Several additional cDNA fragments larger than the normal product of 546 bp were observed, indicating that aberrant splicing had taken place (Fig. 1b).

The multiple RT-PCR products were cloned into the pCRII-TOPO plasmid (Invitrogen). DNA sequencing of individual clones revealed that at least two cryptic splice acceptor sites in intron 12 were utilized (Fig. 2a, filled arrowheads). Both sites con-

tained a short pyrimidine-rich sequence followed by a consensus splice acceptor site sequence (cag). Both transcripts were out-of-frame and predicted to result in premature stop codons, and therefore subject to nonsense-mediated mRNA decay. A computer search employing a modification of the algorithm described by Shapiro and Senapathy (1987) identified numerous potential splice acceptor sequences of significant strength within introns 12 and 13 (Table 2). The strength of the two cryptic splice acceptor sites identified experimentally was slightly higher than that of the normal acceptor site in intron 12. Though not all of the PCR products obtained from cells treated with cycloheximide were cloned and sequenced, it is conceivable that these products resulted from utilization of different cryptic splice acceptor sites in the intron.

The consequence of the mutation at the protein level was studied by immunoprecipitation of metabolically labeled fibroblasts. An antibody specific for the $\alpha 3(\text{VI})$ collagen chain was used so that only those $\alpha 1(\text{VI})$ and $\alpha 2(\text{VI})$ collagen chains that are associated with the $\alpha 3(\text{VI})$ chains would be coimmunoprecipitated. The analysis showed markedly reduced amounts of collagen VI protein in the culture medium from the patient fibroblasts as compared to the control fibroblasts (Fig. 2c). Cells from the patient revealed a strong band that migrated at the position of the $\alpha 1(\text{VI})$ collagen, in contrast to a broad band corresponding to equal amounts of the partially resolved $\alpha 1(\text{VI})$ and $\alpha 2(\text{VI})$ chains found in the control. The $\alpha 2(\text{VI})$ chain normally migrates somewhat more slowly than the $\alpha 1(\text{VI})$ chain under this gel separation condition (Pan et al. 2003). Together, the results suggest that the small amount of the normal $\alpha 2(\text{VI})$ chains produced by the patient's fibroblasts were rapidly assembled and secreted extracellularly. The strong band in the cells from the patient might consist of both the normal $\alpha 1(\text{VI})$ chain and the

Table 1 Nucleotide changes in collagen VI genes

Gene	Exon	Nucleotide change	Amino acid change	Status	Polymorphism frequency ^a
<i>COL6A1</i>	35	2549G → A	R850H	Homozygous	37/108 chromosomes
<i>COL6A1</i>	35	2667G → A	A889A	Heterozygous	39/110 chromosomes
<i>COL6A1</i>	35	2796C → T	S932S	Homozygous	40/112 chromosomes
<i>COL6A2</i>	26	2094G → A	A698A	Homozygous	68/154 chromosomes
<i>COL6A2</i>	26	2097C → T	G699G	Homozygous	68/154 chromosomes
<i>COL6A2</i>	26	2184G → A	V728V	Homozygous	61/154 chromosomes
<i>COL6A2</i>	28	2697T → G	T889T	Homozygous	4/150 chromosomes
<i>COL6A3</i>	28	6855G → C	G2285G	Heterozygous	77/158 chromosomes
<i>COL6A3</i>	36	7596G → A	K2532K	Heterozygous	17/156 chromosomes
<i>COL6A3</i>	39	8491G → C	D2831H	Heterozygous	15/156 chromosomes
<i>COL6A3</i>	40	8780C → T	T2927M	Heterozygous	66/154 chromosomes
<i>COL6A3</i>	40	8820A → G	T2940T	Homozygous	12/156 chromosomes
<i>COL6A3</i>	40	9031C → G	P3011A	Heterozygous	42/156 chromosomes

The reference sequences for the three collagen VI mRNAs are NM_001848 for *COL6A1*, NM_001849 for *COL6A2*, and NM_004369 for *COL6A3* (NCBI database). Nucleotide positions are numbered based on +1 being the A of the translation start codon

^aData from Lampe et al. (2005)

mutant $\alpha 2(\text{VI})$ chain containing the internal exon 13 deletion of 21 amino acids.

Discussion

By mutation, mRNA, and protein analyses, we demonstrate that a homozygous mutation at position -10 in intron 12 of *COL6A2* can cause UCMD. Nucleotide position -10 of splice acceptor sites is not strictly conserved. Although C and T are the preferred nucleotides for this position, A and G are each found approximately 10% of the time in human genes (Zhang 1998). Hence, the A \rightarrow G change detected in the genomic DNA normally would not be considered pathogenic. On the other hand, computer analysis indicates that the strength of the splice acceptor site of intron 12 is relatively weak (score 83.5) compared to those of introns 11, 13, and 14 (scores 91.0–93.5), and

that introns 12 and 13 contain several potential alternative splice acceptor sites with a strength comparable to the normal splice acceptor site (Table 2). It is conceivable that the single base change at -10 disrupts the relatively weak interaction of the intron 12 acceptor site and the splicing machinery, resulting in the activation of the cryptic splice acceptor sites. Most of the aberrant transcripts are degraded by the nonsense-mediated decay mechanism as evidenced by northern analysis, whereas the correctly spliced *COL6A2* mRNA and the transcript with an in-frame deletion of exon 13 remain intact. If mutation screening had been performed by RT-PCR analysis of the mRNA alone, detection of normal and exon 13-deleted *COL6A3* mRNA would be misinterpreted as resulting from a heterozygous splice junction mutation.

The mutant $\alpha 2(\text{VI})$ collagen chain contains a small in-frame deletion in the N-terminus of the triple-helical domain, so it is most likely able to incorporate into a triple-helical monomer (Pan et al. 2003). It also preserves the key cysteine residue needed for maintaining the stability of dimers, and therefore the mutant monomer is predicted to undergo dimer and tetramer formation and be secreted. With a misaligned N-terminal triple-helical region, the abnormal tetramer likely will exert a dominant negative effect on the end-to-end assembly into microfibrils. Indeed, though the biosynthetic study indicates that some collagen VI tetramers are secreted extracellularly, few collagen VI microfibrils can be detected by immunofluorescence, consistent with the notion that the mutant tetramers interfere with microfibrillar formation. Because most of the abnormal transcripts are degraded and only a small amount of the mutant chain is produced, the mutation most likely will not have a striking dominant effect in the heterozygous condition. Indeed, the patient's mother, who is a heterozygous carrier of the mutation, does not display any clinical abnormality.

Predicting splice defects based on sequencing genomic DNA is rather difficult. Moreover, missense changes in exons also may disrupt exon enhancer sequences, thereby causing defects in splicing. Our study demonstrates that in order to understand the precise mechanism of a mutation, both quantitative and qualitative analyses of the mRNA are necessary. The unconventional splicing defect described here presents a challenge to the molecular diagnosis of UCMD, which is already fairly complex because of the possibility of either recessive or dominant mutations. The results highlight the importance of combined molecular and functional analyses in accurate interpretation of genotype-phenotype correlations, a prerequisite for the development of therapeutic strategies.

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Table 2 Potential splice acceptor sites in introns 12 and 13 of *COL6A2*

Nucleotide position ^a	Potential splice acceptor sequence ^b	Score ^c	Comment
283–297	cgetgctcaccgag g	73.8	
312–326	ccacctctccag g	91.0	
318–332	cctcccaggaggag c	66.4	
376–390	cgttggtgccccag g	84.5	
498–512	ccacctccctcag c	83.6	Site used in 2 cDNA clones
504–518	tcctcagcccaag c	71.6	
529–543	ctgagtcctctgag c	71.0	
532–546	agtctctgagcag a	80.2	
540–564	gagcagatgcacag g	68.1	
573–597	cactctgccctcag c	84.1	
594–608	ggaggttcagcag c	67.8	
601–615	ccagcagccccag c	72.2	
605–619	cagccccagccag c	72.8	
681–695	tctgccccgcag c	86.4	Site used in 2 cDNA clones
745–759	gcaaacccttcag G	83.5	Intron 12 acceptor site
764–778	CCCTGGCCGCCAG G	85.0	
836–850	ctcttcgctcag c	87.7	
868–882	cgcccatggcccag a	74.8	
1004–1018	gcctctcagcag g	77.1	
1020–1034	tgaacaagttcag c	74.2	
1032–1046	aggggaccctcag g	74.2	
1101–1115	cacacaccgcacag c	78.6	
1161–1175	Ctctcttcttcag G	93.5	Intron 13 acceptor site
1277–1291	tgcccctgcctcag c	88.3	

^a Nucleotide sequence of the genomic region analyzed is shown in Fig. 2a

^b Potential splice acceptor sites analyzed using the computer program at <http://www.genet.sickkids.on.ca/~ali/splicesitefinder.html>, which employs an algorithm modified from Shapiro and Senapathy (1987). Intron sequences are shown in lower case letters, and exon sequences in capital letters. Vertical lines mark intron/exon borders

^c Strength of the splice acceptor sites calculated using the above computer program

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