Published in final edited form as:

Hum Mutat. 2009 June ; 30(6): . doi:10.1002/humu.21000.

Molecular Mechanisms of Classical Ehlers-Danlos Syndrome

Anna L. Mitchell^{1,*4}, Ulrike Schwarze², Jessica F. Jennings², and Peter H. Byers^{2,3}

¹Department of Pediatrics, Division of Genetics, University of Washington

²Department of Pathology, University of Washington

³Department of Medicine, Division of Medical Genetics, University of Washington

Abstract

Classical Ehlers-Danlos syndrome (EDS) is a heritable disorder characterized by joint hypermobility, skin hyperextensibility, and abnormal wound healing. The majority of affected individuals have alterations in one of the two type V collagen genes, *COL5A1* and *COL5A2*. The most common mechanism is *COL5A1* haploinsufficiency due to instability of the transcript of one allele. In dermal fibroblasts from our population of 76 individuals with clinical features of classical EDS, there were 21 (29.5%) with decreased expression of one *COL5A1* allele, consistent with published estimates of the frequency of null alleles. We identified the causative mutation in 9 of these cell strains (mutations for 7 others had been previously described), and found two nonsense mutations, five splice mutations, and two insertion/deletions. The same type of genomic change at splice sites can have different effects at the RNA level and the outcome could not be predicted from the primary genomic DNA alteration.

Keywords

classical Ehlers-Danlos syndrome; EDS; type V collagen; *COL5A1*; *COL5A2*; haploinsufficiency; G530S

INTRODUCTION

Ehlers-Danlos syndrome (EDS) is a heterogeneous group of inherited disorders characterized by soft, hyperextensible skin, hypermobile joints, abnormal scarring, and other systemic involvement. EDS is divided into several types, based on the clinical features, mode of inheritance, and the underlying genetic basis of each (Beighton et al., 1998; Byers and Schwarze, 2002; Steinmann et al., 2002). Classical EDS (MIM#s 130000, 130010), formerly known as EDS types I/II, is characterized by skin hyperextensibility, widened atrophic (cigarette paper) scars, and large and small joint hypermobility (Beighton et al., 1998). Usually classical EDS results from mutations in the *COL5A1* (MIM# 120215) and *COL5A2* genes (MIM# 120190), which encode the pro 1(V) chain and pro 2(V) chain respectively, of type V collagen. These chains usually combine in a heterotrimer composed of two pro 1(V) and a single pro 2(V) chain. Multiple mutations have been identified in both genes, including substitutions for glycine residues in the triple helical domains, nonsense mutations, insertions/deletions leading to frameshifts, splice site mutations, and a 9-X translocation that disrupts the *COL5A1* gene (Nicholls et al. 1996; Toriello et al., 1996;

*To whom correspondence should be addressed: Anna L. Mitchell, Case Western Reserve University, Center for Human Genetics, 11100 Euclid Avenue, Lakeside 1500, Cleveland, OH 44106-5000; alm43@case.edu.

⁴current address: Department of Genetics, Case Western Reserve University

Supporting Information for this preprint is available from the *Human Mutation* editorial office upon request (humu@wiley.com)

Wenstrup et al. 1996; De Paepe et al 1997; Burrows et al. 1998; Michalickova et al., 1998; Richards et al., 1998; Malfait et al, 2005). Approximately 25–30% of individuals with classical EDS are haploinsufficient for the *COL5A1* mRNA as a consequence of mutations that lead to nonsense-mediated mRNA decay (Schwarze et al., 2000; Wenstrup et al, 2000; Malfait et al., 2005).

Premature stop codons (PTCs) can appear as a consequence of nonsense mutations, insertion/deletions that alter the reading frame, or splice site changes that lead to the use of cryptic splice sites and an altered reading frame. For nonsense mediated mRNA decay (NMD) to occur, the PTC is generally upstream of the last intron and separated from that intron by at least fifty basepairs (Neu-Yilik et al., 2004).

Because *COL5A1* haploinsufficiency accounts for a significant fraction of mutations in classical EDS cases, we identified individuals with decreased expression of one allele, identified the causative mutations in a subset of them, and determined how these mutations altered the stability of the resulting mRNA molecules.

MATERIALS AND METHODS

Subject Selection

We selected 76 study subjects, which included the 16 previously described (Schwarze et al., 2000), from the University of Washington Collagen Diagnostic Laboratory Repository of individuals referred for evaluation for EDS. Subjects were included if they had all three of the major diagnostic criteria for classical EDS as defined by Beighton et al., (1998): hypermobile joints, hyperextensible skin, and characteristic scars or other evidence of poor wound healing. Samples were obtained with appropriate consent and the Human Subjects Division at the University of Washington approved the study.

Cell Culture; Isolation of DNA and RNA; Protein Analysis

A punch skin biopsy or cultured dermal fibroblasts were provided for each subject. If a skin biopsy was received, fibroblasts were cultured as described previously (Bonadio et al., 1985). DNA and RNA were isolated using the appropriate Qiagen kit (QIAamp DNA Mini kit; RNeasy Mini kit; Qiagen, Valencia, CA). Complementary DNA was synthesized from the RNA using the Superscript kit (Gibco BRL).

Collagenous proteins were labeled and studied as previously described (Bonadio et al., 1985).

Assessment of *COL5A1* expression

To determine if both *COL5A1* alleles were expressed at equal levels, genomic DNA from each cell strain was screened at four common polymorphic sites in the coding sequence and in the 3' untranslated region (Schwarze et al., 2000). After identification of a heterozygous site, cDNA was PCR-amplified with primers spanning the variant region, and the product was digested with the appropriate enzyme before analysis by polyacrylamide gel electrophoresis (Schwarze et al., 2000). Eight of the samples with complete or partial haploinsufficiency were selected for analysis of genomic DNA to identify the causative mutation. One cell strain in which the four markers were homozygous was also evaluated at the genomic level.

Amplification and sequencing of cDNA and genomic DNA

COL5A1 cDNA was PCR-amplified in six overlapping, large fragments (Schwarze et al., 2000) and the products were analyzed on a 7% polyacrylamide gel. PCR products of the

expected size were sequenced using the Big Dye terminator method on an ABI310 Genetic Analyzer (Applied Biosystems) to identify missense mutations (reference sequence=RefSeq NM_000093.2; numbered with reference to A of initiation ATG as 1). When a novel band was also detected on the gel, the individual exons within that fragment were amplified from genomic DNA.

Sixty-five of the sixty-six exons of the *COL5A1* gene were PCR-amplified from genomic DNA in fifty-seven reactions. Sequences of the primers and amplification conditions used are listed in Supp. Table S1. Buffer composition and times for steps of PCR are as noted in attachment 2. PCR products were sequenced using the Big Dye terminator method and the ABI310 (Applied Biosystems).

The *COL5A2* cDNA was PCR-amplified in five overlapping fragments (Schwarze et al., 2000). PCR products were sequenced with the Big Dye terminator method on the ABI310 (Applied Biosystems) (reference sequence= RefSeq NM_000393.2; numbered with reference to A of initiation ATG as 1).

Genomic heteroduplex screen of *COL5A1*

Sixty-five of the 66 exons of the *COL5A1* gene (exon 1 was omitted) were amplified in twenty multiplex reactions and three single exon amplifications. Multiplex combinations and amplification conditions are listed in Supp. Table S2. Following amplification, thermocycler conditions were set to favor heteroduplex formation. Products were analyzed on mutation enhancement detection (MDE) gels (BioWhittaker Molecular Applications), and visualized after staining with ethidium bromide. Products with heteroduplexes were individually re-amplified and sequenced with the Big Dye terminator method on an ABI310 to identify the source of the alteration.

Isolation of nuclear RNA

Skin fibroblasts were grown to confluence in a 100mm dish, harvested, and centrifuged to isolate the nuclear pellet. Nuclei were lysed and RNA collected following the protocol of the RNeasy Minikit (Qiagen, Valencia, CA) (nuclear RNA [nRNA] fraction). RNA was also isolated from the cytoplasmic fraction using the same kit (cytoplasmic RNA [cRNA] fraction). These two RNA fractions were separately treated with RNase-free DNase at 37° for 1 hour prior to being used as templates for cDNA synthesis (Superscript, GibcoBRL).

Screening of nuclear and cytoplasmic RNA

To identify novel products expressed from the mutant allele, cDNA was synthesized using the nuclear and cytoplasmic RNA templates, with primers located in exons spanning the region of the identified mutations (Supp. Table S3). Products were analyzed on 7% polyacrylamide gels stained with ethidium bromide. Novel bands were cut from the gel, re-amplified with the initial amplification primers, purified with the QIAquick-spin columns (Qiagen, Valencia, CA), and sequenced with the Big Dye terminator method on the ABI310 (Applied Biosystems).

To distinguish normal and mutant products that differed from each other by only one or two basepairs, restriction digestion was done (P6 and P7). Either *BsaI* (P6) or *TspRI* (P7) was incubated with PCR product at 65° overnight, followed by analysis on 7% polyacrylamide gel. The mutation in P1 did not affect a restriction site, so a new PCR primer (x49mm; 5'-CTTCTCCCGGAAGGCAGACT-3') was designed to introduce a *HinfI* site in the normal *COL5A1* sequence. The novel restriction site is present only in the product of the normal allele. These PCR products were digested overnight at 37°.

Screening for exon 13 variant

To screen for the c.1588G>A (p.Gly530Ser) variant in *COL5A1*, genomic DNA was PCR-amplified using primers in introns 11 (in11S: 5'-CTGGCCTGGTTGCACTCTGACTTG-3') and 13 (in13A: 5'-GGCTGGGCTCTGTCCCCTTCAGG-3') (denaturation at 95° for 2 minutes, 35 cycles of 95° for 15 sec, 62° for 20 sec, 72° for 30 sec and a final extension at 72° for 5 minutes). PCR products were digested overnight with restriction endonuclease *BbvI* at 37°, and analyzed on 7% polyacrylamide gel. There are three constitutive *BbvI* sites within the PCR product at positions 104 (exon 12), 167 and 181 (intron 12). The site created by the variant is at position 325 (exon 13). Samples were screened from individuals with connective tissue disorders: 72 with classical EDS; 94 with vascular EDS; 82 with osteogenesis imperfecta, as well as anonymous control samples: 49 with neurofibromatosis I; 50 being evaluated for fragile X syndrome; and 51 referred for hemochromatosis testing.

RESULTS

Stable expression of *COL5A1* alleles

Of seventy-six individuals screened for heterozygosity at each of four polymorphic sites in the *COL5A1* gene, seventy (92%) were heterozygous at one or more sites. In cells from twenty of the seventy individuals (28.5%), the mRNA product of one *COL5A1* allele was absent or decreased. Cells from eight of these individuals were selected for further study to identify the causative mutation (mutations for 7 of the 20 individuals were reported by Schwarze et al., 2000 and Takahara et al., 2002).

Analysis of *COL5A1* cDNA from patients with null alleles

For four cell strains (P1, P8, P11, and P12), amplification of one or more of the *COL5A1* cDNA fragments produced a faster-migrating band or a heteroduplex, in addition to the expected band (Figure 1). Sequencing of the genomic DNA in the regions that contained the heteroduplexes led to identification of the genomic mutations: c.2844+1G>A (IVS35+1G>A) (P11); c.2845-2A>C (IVS35-2A>C) (P12); c.3874G>A, p.Glu1292Lys in exon 49 (P1); and exon 62 c.4916_4931del16 (P8). Each of these mutations resulted in creation of a frameshift, a downstream PTC and mRNA instability in the product of the mutant allele (see below).

Analysis of *COL5A1* gDNA from patients with null alleles

Heteroduplex analysis of *COL5A1* genomic DNA followed by sequence determination of the abnormal fragment from the four cell strains (P5, P6, P9, and P10) with haploinsufficiency, but no abnormality detected by cDNA screening identified four additional mutations: exon 4, c.495G>A; p.Trp165X (P5); exon 13, c.1654C>T; p.Glu552X (P6); c.1989+1G>A (IVS19+1G>A) (P9); and c.2386-1G>A (IVS27-1G>A) (P10).

Heteroduplex analysis of genomic DNA from one individual (P7) uninformative for the markers used in the haploinsufficiency screen led to identification of a mutation (c.3680_3681insT in exon 46), which leads to a premature termination codon in exon 47. The mRNA from the mutant allele was rapidly degraded so that virtually no stable product was present in the cytoplasm (Figure 2A; lane 3).

Characterization of products from use of cryptic splice sites

In P9 cells (c.1989+1G>A; IVS19+1G>A), the mutant allele used a cryptic splice site four nucleotides downstream in intron 19. The inclusion of four nucleotides from intron 19 altered the reading frame, and resulted in a PTC in exon 20, and a message that should be subject to NMD. A small amount of heteroduplex was visible in the cytoplasmic RNA (lane

2, Figure 3A), which could indicate that not all the message was degraded prior to entering the cytoplasm or that there was incomplete separation of the nuclear fraction from the cytoplasm during isolation of the RNA. A +1G>A alteration in another intron, intron 35 (c. 2844+1G>A; IVS35+1G>A; P11), resulted in two splice outcomes- use of a cryptic intronic splice site with partial intron inclusion, and exon 35 skip (Figure 3B). The addition of 118 nucleotides of intron 35 altered the reading frame and created a premature termination codon in exon 39. This product is subject to NMD, while the skip of the 45 nt exon 35 does not alter the reading frame and the mRNA is stable (lane 1 vs. lane 2, Figure 3B).

With a splice acceptor mutation in intron 35 (c.2845-2A>C; IVS35-2A>C; P12), there were also two outcomes- use of a cryptic acceptor site within exon 36 so that 19 nucleotides of coding sequence were lost, and skip of exon 36 (lane 1, Figure 3C). Both products are in relatively low abundance suggesting that, because the exon-skip product is stable, the major outcome of the mutation was the use of the cryptic acceptor site which created a PTC in exon 41, leading to NMD.

With the splice acceptor site mutation c.2386-1G>A (IVS27-1G>A; P10), a shift of the splice site one nucleotide downstream resulted in a PTC within exon 28. A heteroduplex, visible only after amplification of the nuclear RNA (lane 1, Figure 3D), was not seen in the cytoplasmic RNA (lane 2, Figure 3D).

Insertion of a single nucleotide altered the reading frame, and resulted in an unstable message, expected to undergo NMD in cells from P7 (c.3680_3681insT). In the nucleus, there are approximately equal amounts of normal and mutant messages (Figure 2A, lane 2). In the cytoplasm, close to 99% of the product is cut by *TspRI*, a site present only in the product of the normal allele (Figure 2A, lane 3).

Deletion of sixteen nucleotides from exon 62 (P8) led to a frameshift and PTC in exon 65. The message from the abnormal allele was detected in RNA from the nucleus, but not in the cytoplasmic RNA (Figure 2B, lane 1 vs. lane 2). Again, this demonstrates the degree of message degradation.

In cells from P6, there were no novel bands present after amplification of both nuclear and cytoplasmic RNA fractions. Three different primer sets spanning the region of the mutation were tried and in all cases, the only band visible was that of the expected size. Because the mutation creates a *BsrI* site, the normal allele can be differentiated from the mutant one by restriction enzyme digestion. In nuclear RNA, approximately 40% of the RT-PCR product was cut twice by *BsrI*, once at a constitutive site and once at a site introduced by the mutation (lane 1, Figure 2C), while the majority of the product from cytoplasmic RNA was cut at only the constitutive site (lane 2, Figure 2C), consistent with loss of the mutant product by NMD.

In cells from P1 (exon 49 c.3874G>A, p.Glu1292Lys) the missense mutation in exon 49 reduced mRNA stability. RT-PCR of nuclear RNA revealed a novel, smaller product (lane 1, Figure 4A), that resulted from use of a new donor splice site in exon 49 formed by the mutation. The loss of thirty-five basepairs from exon 49 created a PTC in exon 50. This product is not detected in RNA from the cytoplasm, indicating that it has all been degraded (lane 2, Figure 4A). The cryptic exon 49 sequence used for splicing is AGGTAAGC (altered nucleotide underlined), which differs from the consensus splice donor site [AGgt(a/g)agt] by a single nucleotide. The Shapiro and Senapathy score (Shapiro and Senapathy, 1987) for the constitutive sequence (CGgtgagt) is 88.5, while that for the cryptic sequence is 94.2, consistent with the preference for the cryptic site by the splicing machinery.

The second product expressed from the mutant allele is identical in length to the product of the normal allele, but differs by one nucleotide (A vs G). Amplification with a mismatched primer, designed to introduce a *HinA* site in the normal sequence, resulted in identical amplification products for the patient and a control. The mismatched reverse primer is located in the part of exon 49 that is deleted by the use of the cryptic splice site so there is no amplification of mRNA containing the deletion. After incubation with *HinA*, the control samples are cut completely (lanes 3 and 4, Figure 4B), while the patient samples are only partially digested (lanes 1 and 2, Figure 4B).

Sequencing of *COL5A1* cDNA from previous study subjects

COL5A1 cDNA was amplified in six overlapping large fragments and sequenced from two of the eight individuals who did not have null alleles in our previous publication (P9 and P15, Schwarze et al, 2000; P3 and P2, respectively, in this study). Missense mutations were identified in both: P2 c.4465G>A, p.Gly1489Arg, Gly931Arg in the triple helix and P3 c.5504G>C, p.Cys1835Ser in the carboxyl-terminal propeptide. This cysteine is the last cysteine of the carboxyl-terminal propeptide. In all fibrillar collagen pro chains, this residue is important for the stabilization of an intramolecular disulfide bond that is necessary for chain association. Substitution for that cysteine usually results in failure of chain incorporation into trimers. These sequence alterations were confirmed in genomic DNA.

Abnormality of $\alpha 2(V)$ in one subject

We identified an abnormality of type V collagen by protein screening in only one individual (P4). The $\alpha 2(V)$ band in the cell layer proteins was broadened, consistent with overmodification of the protein (Figure 5). The type V collagen produced by cells from the remaining 75 individuals could not be distinguished from those made by control cells.

Sequencing of *COL5A2* cDNA

A missense mutation (c.3437G>C; p.Gly1149Ala; Gly937Ala in triple helix) was identified by sequencing of *COL5A2* cDNA from the individual with the protein gel abnormality. The mutation created an *EaeI* site so its presence was confirmed in genomic DNA, both by sequencing of the relevant exon (46) and by restriction digestion of an exon 46 PCR product. The alteration was not present in DNA from either unaffected parent.

Exon 13 variant

Six of the 72 individuals with classical EDS were heterozygous for the exon 13 c.1588G>A (p.Gly530Ser) variant in *COL5A1* (6/144 alleles= 4.2%; Table 1). This is similar to the fraction of the variant allele among individuals with vascular EDS (8/188=4.2%), and individuals with OI (9/164=5.5%). Among the control samples, the frequency of the variant allele ranged from 3.1% to 9.8%. Only one homozygote was detected among the 398 individuals evaluated and it was in an anonymous control sample. This variant is not listed in dbSNP, the reason for which is unclear, given these frequencies.

DISCUSSION

Mutations in the type V collagen genes, *COL5A1* and *COL5A2*, lead to the phenotype of classical EDS. Previously it has been shown that 25–30% of individuals with classical EDS have mutations that decrease the stability of the messenger RNA expressed from one *COL5A1* allele (Schwarze et al. 2000, Wenstrup et al., 2000; Malfait et al., 2005). In our screen of seventy-six subjects with phenotypes consistent with classical EDS, we found that twenty of the seventy who were informative for the markers used had decreased or absent sequences from one *COL5A1* allele. In addition, one of the six individuals uninformative for

all markers also had a mutation that resulted in a null allele (total nulls=21; 21/71=29.5%). Cells from some individuals were completely lacking mRNA from one *COL5A1* allele (nulls), while others had decreased (but not absent) amounts of mRNA from one allele (partial nulls).

We previously described the genomic mutations for seven of the twenty-one individuals with null or partial null alleles (Schwarze et al. 2000, Takahara et al, 2002). We now have identified genomic alterations in nine of the remaining fourteen individuals. Five had mutations affecting splicing (four intronic, one exonic), two had nonsense mutations, and two had insertions/deletions that resulted in reading frame shifts and downstream premature termination codons (Table 2). In each of the nine, the genomic alteration resulted in creation of a premature stop codon, leading to nonsense-mediated decay of the message containing it.

For the three nonsense and insertion/deletion mutations for which samples were available (P6, P7, and P8; P5 not studied), there was only one product expressed from each mutant allele and all message was subjected to NMD.

The effects of splice site mutations were varied. Some resulted in a single outcome and an mRNA with a PTC that underwent NMD (P9 and P10). Other splice site mutations led to two different products (P11 and P12). In these cases, one product contained a PTC and was degraded. The other product of each mutant allele had an intact reading frame, thereby providing stability and protection from NMD.

Outcomes of splice site mutations often cannot be predicted from the change in genomic DNA, as shown by the comparison of outcomes for P9 and P11, both of which have an intronic +1G>A donor site mutation. In one case (IVS19, P9), the only detectable outcome of the mutation was use of a cryptic intronic splice site, leading to partial intron inclusion, frameshift with a PTC in exon 20, and mRNA degradation (Figure 3A). In the other case (IVS35, P11), there were two outcomes. One was partial intron inclusion, as occurred in the previous example and which created an unstable mRNA. The second product resulted from skipping of a forty-five basepair exon, which maintained an intact reading frame (Figure 3B).

These findings indicate that different transcripts of the same allele may adopt different conformations or be processed in different ways. Outcomes of splice site mutations reflect characteristics of the surrounding sequences- the presence of cryptic splice sites that can be activated, and whether the use of a new splice site results in an in-frame or out-of-frame transcript. These possibilities do not account for the presence of more than one outcome, which might be explained, in part, by the local order of intron removal in the region of the mutation (Schwarze et al., 1999). If the intron just prior to one with a donor site mutation is removed more slowly than the one with the mutation, and its removal is dependent upon the splicing of the downstream intron (in this case the affected one), then the cell will retain both introns until the precursor RNA refolds so that splicing results in skipping of the exon adjacent to the mutation. If however, the upstream intron is removed more quickly than the one with the donor site mutation, then exon skipping is not possible and use of a cryptic exonic or intronic splice site downstream of the native one, or whole intron inclusion are the possible outcomes with mRNA stability dependent on whether the product is in or out of frame. The presence of different outcomes from the same allele suggests that in the wild type allele, the folding of the nascent mRNA may differ among transcripts and facilitates the use of different splice pathways. Then, in the presence of a mutation, these splice pathways lead to different outcomes.

Missense Mutations

Substitutions of glycine residues in the triple helical domain of fibrillar collagens disrupt the propagation of the collagen triple helix and result in abnormal molecules that can be disease-causing. We identified two patients with substitutions for glycine as the mechanism of their classical EDS, one each in *COL5A1* and *COL5A2*. The only previously published example of a substitution for glycine in *COL5A2* involved the same glycine residue (1149), but the G>C transversion in the first position of the codon resulted in substitution by arginine (Richards et al., 1998) rather than by alanine, as seen here. This alteration was reflected in the electrophoretic mobility shift seen in the $\alpha 2(V)$ chains made by the cultured dermal fibroblasts.

Cysteine residues in the carboxyl-terminal propeptide of fibrillar collagens are involved in inter- and intrachain disulfide bonds. Cysteine is not found in the triple helical domain, presumably because it would increase the likelihood of having unpaired residues. There are eight cysteine residues in the carboxyl-terminal propeptide of the pro $\alpha 1(V)$ chain, the first four of which form interchain disulfide bonds that stabilize the trimeric structure. The last four form a loop within a loop structure that allows a chain recognition site to appear on each chain. The substituted residue is the last cysteine of the chain and its absence in other fibrillar collagen chains leads to failure of incorporation and chain degradation. This appears to be another mechanism for “haploinsufficiency”, but at the protein level. The abnormal protein would be rapidly degraded.

Exon 13 Variant

The c.1588G>A p.Gly530Ser variant in *COL5A1* gene has been described previously in individuals with classical EDS (Giunta and Steinmann, 2000; Giunta et al, 2002) and proposed to be disease-modifying in the heterozygous state and disease-causing when homozygous. Although not found in dbSNP, we found that the frequency of the allele with the variant A was similar (about 5%) in groups with classical EDS, vascular EDS, and OI, and in three control groups we tested. Hardy-Weinberg equilibrium predicts the frequency of individuals homozygous for the variant to be 1/400, using an allele frequency of 1/20 (5%). This is consistent with the data obtained here, as there was one homozygote among the 398 individuals screened. If homozygosity for the variant A led to classical EDS, then about 1/400 individuals would be affected. The frequency of the classical form of EDS is thought to be far lower than that (although good epidemiological data are not available) so that homozygosity for the exon 13 variant is unlikely to cause this condition.

From the small number of individuals studied here, it was not possible to determine if there are any significant differences in phenotype among those that have different classes of mutations in the type V collagen genes (Table 2).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

We thank Diana Chen, Suzanne Mooney, and Emily Yim for technical assistance with the protein gels, and LuAnn Judis for assistance with figures. This work was supported by grants AR21557, 2 T32 GM 007454-31, AR051444 and HD043376.

References

- Beighton P, De Paepe A, Steinmann B, Tsipouras P, Wenstrup RJ. Ehlers-Danlos syndromes: revised nosology, Villefranche, 1997. Ehlers-Danlos National Foundation (USA) and Ehlers-Danlos Support Group (UK). *Am J Med Genet.* 1998; 77:31–7. [PubMed: 9557891]
- Bonadio J, Holbrook KA, Gelinis RE, Jacob J, Byers PH. Altered triple helical structure of type I procollagen in lethal perinatal osteogenesis imperfecta. *J Biol Chem.* 1985; 260:1734–1742. [PubMed: 2981871]
- Burrows NP, Nicholls AC, Richards AJ, Luccarini C, Harrison JB, Yates JRW, Pope FM. A Point Mutation in an Intronic Branch Site Results in Aberrant Splicing of *COL5A1* and in Ehlers-Danlos Syndrome Type II in Two British Families. *Am J Hum Genet.* 1998; 63:390–398. [PubMed: 9683580]
- Byers, PH.; Schwarze, U. Ehlers-Danlos syndrome. In: Rimoin, DL.; Connor, JM.; Pyeritz, RE.; Korf, B., editors. *Emery and Rimoin's Principles and Practice of Medical Genetics.* Churchill Livingstone; London: 2002. p. 4021–4043.
- DePaepe A, Nuytinck L, Hausser I, Anton-Lamprecht I, Naeyaert JM. Mutations in the *COL5A1* Gene Are Causal in the Ehlers-Danlos Syndromes I and II. *Am J Hum Genet.* 1997; 60:547–554. [PubMed: 9042913]
- Giunta C, Steinmann B. Compound Heterozygosity for a Disease-Causing G1489D and Disease-Modifying G530S Substitution in *COL5A1* of a Patient with the Classical Type of Ehlers-Danlos Syndrome: An Explanation of Intrafamilial Variability? *Am J Med Genet.* 2000; 90:72–79. [PubMed: 10602121]
- Giunta C, Nuytinck L, Raghunath M, De Paepe A, Steinmann B. Homozygous Gly530Ser substitution in *COL5A1* causes mild classical Ehlers-Danlos syndrome. *Am J Med Genet.* 2002; 109(4):284–290. [PubMed: 11992482]
- Malfait F, Coucke P, Symoens S, Loeys B, Nuytinck L, De Paepe A. The molecular basis of classic Ehlers-Danlos syndrome: a comprehensive study of biochemical and molecular findings in 48 unrelated patients. *Hum Mutat.* 2005; 25(1):28–37. [PubMed: 15580559]
- Michalickova K, Susic M, Willing MC, Wenstrup RJ, Cole WG. Mutations of the $\alpha 2(V)$ chain of type V collagen impair matrix assembly and produce Ehlers-Danlos syndrome type I. *Hum Mol Genet.* 1998; 7(2):249–255. [PubMed: 9425231]
- Nicholls AC, Oliver JE, McCarron S, Harrison JB, Greenspan DS, Pope FM. An exon skipping mutation of a type V collagen gene (*COL5A1*) in Ehlers-Danlos syndrome. *J Med Genet.* 1996; 33:960–946.
- Neu-Yilik G, Gehring NH, Hentze MW, Kulozik AE. Nonsense-mediated mRNA decay: from vacuum cleaner to Swiss army knife. *Genome Biol.* 2004; 5(4):218. [PubMed: 15059251]
- Richards AJ, Martin S, Nicholls AC, Harrison JB, Pope FM, Burrows NP. A single base mutation in *COL5A2* causes Ehlers-Danlos syndrome type II. *J Med Genet.* 1998; 35:846–848. [PubMed: 9783710]
- Schwarze U, Starman BJ, Byers PH. Redefinition of Exon 7 in the *COL1A1* Gene of Type I Collagen by an Intron 8 Splice-Donor-Site Mutation in a Form of Osteogenesis Imperfecta: Influence of Intron Splice Order on Outcome of Splice-Site Mutation. *Am J Hum Genet.* 1999; 65:336–344. [PubMed: 10417276]
- Schwarze U, Atkinson M, Hoffman GG, Greenspan DS, Byers PH. Null Alleles of the *COL5A1* Gene of Type V Collagen Are a Cause of the Classical Forms of Ehlers-Danlos Syndrome (Types I and II). *Am J Hum Genet.* 2000; 66:1757–1765. [PubMed: 10796876]
- Shapiro MB, Senapathy P. RNA splice junctions of different classes of eukaryotes: sequence statistics and functional implications in gene expression. *Nucleic Acids Res.* 1987; 15(17):7155–7174. [PubMed: 3658675]
- Steinmann, B.; Royce, PM.; Superti-Furga, A. The Ehlers-Danlos Syndrome. In: Royce, PM.; Steinmann, B., editors. *Connective Tissue and its Heritable Disorders. Molecular, Genetic and Medical Aspects.* 2. Wiley-Liss; New York: 2002. p. 35–407.
- Takahara K, Schwarze U, Imamura Y, Hoffman GG, Toriello H, Smith LT, Byers PH, Greenspan DS. Order of intron removal influences multiple splice outcomes, including a two-exon skip, in a

- COL5A1 acceptor-site mutation that results in abnormal pro- α 1(V) N-propeptides and Ehlers-Danlos syndrome type I. *Am J Hum Genet.* 2002; 71(3):451–465. [PubMed: 12145749]
- Toriello HV, Glover TW, Takahara K, Byers PH, Miller DE, Higgins JV, Greenspan DS. A translocation interrupts the *COL5A1* gene in a patient with Ehlers-Danlos syndrome and hypomelanosis of Ito. *Nat Genet.* 1996; 13:361–365. [PubMed: 8673139]
- Wenstrup RJ, Langland GT, Willing MC, D'Souza VN, Cole WG. A splice-junction mutation in the region of *COL5A1* that codes for the carboxyl propeptide of pro 1(V) chains results in the *gravis* form of the Ehlers-Danlos syndrome (type I). *Hum Mol Genet.* 1996; 5(11):1733–1736. [PubMed: 8923000]
- Wenstrup RJ, Florer JB, Willing MC, Giunta C, Steinmann B, Young F, Susic M, Cole WG. *COL5A1* Haploinsufficiency Is a Common Molecular Mechanism Underlying the Classical Form of EDS. *Am J Hum Genet.* 2000; 66:1766–1776. [PubMed: 10777716]

Figure 1.

Amplification of *COL5A1* overlapping large cDNA fragments made from whole cell RNA.

A. P12; amplification of exons 14–37 has a normal fragment and one with faster than expected mobility, as well as a heteroduplex.. **B.** P11; amplification of exons 27–48 has a fragment with normal mobility and a heteroduplex, indicative of a size alteration in some of the fragments of apparently normal size. **C.** P1; amplification of exons 44–57 with a small amount of a smaller than expected product and a heteroduplex. **D.** P8; amplification of exon 60-3 UTR. The heteroduplex in the control results from an alternative splice variant. There is an additional heteroduplex in the subject sample. C= control individual; P= subject; HD= heteroduplex

Figure 2.

Distribution of abnormal allele RNA. **A.** *TspRI* digestion of PCR product from P7 (*COL5A1* exon 46 c.3680_3681insT). Virtually all the uncut product (from the mutant allele) is in the nuclear fraction. **B.** Amplified products from P8 (*COL5A1* c.4916_4931del16). The cDNA was amplified with primers in exons 60 and 63. The expected 391 bp product of exons 60–63 is indicated. The product from the abnormal allele with the 16 basepair deletion is seen only in the nuclear compartment. **C.** P6: (*COL5A1* c.1654C>T; p.Gln552X). The mutation introduces a new *BspI* site in the amplified cDNA. The majority of the abnormal product is in the nucleus with virtually none in the cytoplasmic fraction. wt= wildtype; mut= mutant; n= nuclear RNA; c= cytoplasmic RNA; T=*TspRI* site; B=*BspI* site.

Figure 3.

Effects of *COL5A1* mutations on splicing. **A.** P9: *COL5A1* c.1989+1G>A (IVS19+1G>A); Amplification primers in exons 17 and 21 produced the expected 219 bp product. The product from the abnormal allele results from use of an intronic splice site and inclusion of four nucleotides (atag) from intron 19. The heteroduplex is seen in both nuclear and cytoplasmic samples in low abundance. **B.** P11: *COL5A1* c.2844+1G>A (IVS35+1G>A). Primers in exons 33 and 37 were used and the expected 268 bp product is indicated. The smallest band in lanes 1 and 2 results from skipping of exon 35. The other product from the abnormal allele results from use of an intronic splice site and inclusion of 118 nucleotides from intron 35. The heteroduplex derived from the normal product and the intron inclusion product is seen only with the nuclear material (HD3). **C.** P12: *COL5A1* c.2845-2A>C (IVS35-2A>C). Primers in exons 33 and 37 were used and the expected 268 bp product is indicated. The smallest band in lanes 1 and 2 results from skipping of exon 36. The other product from the abnormal allele results from use of a cryptic exonic splice site and deletion of nineteen nucleotides from exon 36. Heteroduplex 1 (HD1) results from interaction between the normal product and the exon skip product and is seen in the nuclear and cytoplasmic fractions. HD2, which results from interaction between the normal product and the partial exon deletion product, is poorly represented in the cytoplasmic fraction. **D.** P10: *COL5A1* c.2386-1G>A (IVS27-1G>A). Primers in exons 25 and 30 were used and the expected 295 bp product is indicated. The product from the abnormal allele results from the use of a splice site generated by the mutation that is 1 nt downstream and results in deletion

of one nucleotide from exon 28. It is seen almost exclusively in the nuclear fraction.
n=nuclear RNA; c=cytoplasmic RNA; HD=heteroduplex; arrows indicate location of
primers used for PCR-amplification.

A. **B.** **Figure 4.**

Identification of abnormal splice products in cells from P1 (*COL5A1* c.3874G>A; p.Glu1292Lys). **A.** Samples from cDNA were amplified with primers in exons 48 and 54 of *COL5A1*. The expected 447 bp product is indicated. The smaller band, seen only in the nuclear fraction, results from use of a cryptic splice donor site within exon 49 and deletion of all but 19 basepairs of the exon. **B.** Determination of amounts of non-deletion products expressed from each allele. Samples from cDNA synthesized from nuclear and cytoplasmic RNA were PCR-amplified with primers in exons 44 and in the segment of exon 49 deleted as a result of the use of the newly formed donor site. The exon 49 reverse primer differed from the wildtype sequence by 1 nucleotide so that a *HinfI* restriction enzyme site was created with the normal product. The amplification product is 373 bp; 21 bp are removed from the product of the normal allele after digestion. A little less than half the product in the

cytoplasm represents the mutant sequence. n=nuclear RNA; c=cytoplasmic RNA; HD=heteroduplex; H=*Hinf*I site; arrows indicate location of primers used for PCR-amplification.

Figure 5. SDS-PAGE of radiolabeled collagens from P4. The 1(V) and 2(V) bands in the P4 cell layer are broader than expected (lane 4 vs. lane 3), indicating overmodification of the protein. C=control sample; P= patient sample.

Table 1

Allele frequencies at *COL5A1* c.1588G>A, p.Gly530Ser

Source	Individuals	Alleles	A/A Homozygotes	A/G heterozygotes	A allele frequency
EDSI/II	72	144	0	6	4.2%
EDS IV	94	188	0	8	4.2%
OI	82	164	0	9	5.5%
NFI	49	98	0	3	3.1%
Hemochro matosis	51	102	1	8	9.8%
FRAX	50	100	0	5	5%
TOTAL	398	796	1	39	5.00%

Results of screening for G530S allele (c.1588G>A) in different populations. Genomic DNA was amplified with primers in introns 11 and 13 and digested with *BbvI* at 37° for overnight. The numbers of heterozygotes and homozygotes are indicated. On average, the allele frequency in the groups was 5% (1/20). EDS= Ehlers-Danlos syndrome; OI= Osteogenesis imperfecta; NFI= Neurofibromatosis type 1; FRAX= Fragile X syndrome.

Table 2

Summary of mutations identified in patients with classical EDS

Mutation type	Subject	DNA ¹	RNA	protein	age at dx	phenotype
Codon changes	missense	5A1 x49 c.3874G>A	a) missense	a) p.Glu1292Lys	</=34y	velvety, hyperextensible skin; hypermobile joints; unusual/puckering scars
			b) 35bp del x49	b) frameshift PTC x50		
	P2	5A1 x58 c.4465G>A	missense	p.Gly1489Arg	2 y	hypermobile joints; wide, atrophic scars; easy bruising
	P3	5A1 x66 c.5504G>C	missense	p.Cys1835Ser	</=14y	soft, velvety, hyperextensible skin; wide, atrophic scars; hypermobile joints
	P4	5A2 x46 c.3437G>C	missense	p.Gly1146Ala	</=7 y	soft, hyperextensible, skin; cigarette paper scars; easy bruising, hypermobile joints
nonsense	P5	5A1 x4 c.495G>A	nonsense	p.Trp165X	5 y	hyperextensible skin; atrophic skin in pretibial region; scoliosis, flat feet
	P6	5A1 x13 c.1654C>T	nonsense	p.Gln552X	4 y	loose skin; hypermobile, subluxing joints; atrophic scars
Frame-shift	P7	5A1 x46 c.3680_3681insT	1 bp insertion	frameshift PTC x47	</=16y	soft, doughy, hyperextensible skin; cigarette paper scars, hypermobile joints
	P8	5A1 x62 c.4916_4931del	16 bp del	frameshift PTC x65	6 y	soft, velvety hyperextensible skin; slow wound healing; cigarette paper scars; easy bruising; MVP; scoliosis
Splice sites	P9	5A1 c.1989+1G>A (IVS19+1G>A)	4 bp incl IVS19	frameshift PTC x20	</=8y	soft skin; cigarette paper scars; easy bruising; hyperextensible fingers; prominent scars
	P10	5A1 c.2386-1G>A (IVS27-1G>A)	1bp del x28	frameshift PTC x28	8 y	hyperextensible skin; cigarette paper scars; hypermobile joints
	P11	5A1 c.2844+1G>A (IVS35+1G>A)	a) x35 skip	a) x35 skip	2 y	doughy, hyperextensible skin; hypermobile joints; easy bruising
			b) 118bp incl IVS35	b) frameshift PTC x39		
	P12	5A1 c.2845-2A>C (IVS35-2A>C)	a) x36 skip	a) x36 skip	</=31y	soft, velvety, loose skin; poor wound healing; hyperextensible joints; periodontal disease
			b) del 19bp x36	b) frameshift PTC x41		

Information about the patient's age at diagnosis and phenotype is included. If the information from the referring physician indicated the age at which the patient was diagnosed, then that number is included in the table (column 7). When this information was not provided, the age at which the skin biopsy was received in the Collagen Diagnostic Laboratory for analysis was used with "</=" preceding it. Reference sequences= GenBank NM000093.2 (COL5A1) and NM000393.2 (COL5A2).

¹ numbered with reference to the A of initiation ATG as 1. x=exon; IVS= intervening sequence; bp= basepair; ins= insertion, del=deletion; incl= inclusion; MVP= mitral valve prolapse; PTC= premature termination codon; y= years.