RESEARCH ARTICLE

Human Mutation

OFFICIAL JOURNAL
HGVS
HUMAN GENOME VARIATION SOCIETY
www.hgvs.org

Comprehensive Molecular Analysis Demonstrates Type V Collagen Mutations in over 90% of Patients with Classic EDS and Allows to Refine Diagnostic Criteria

Sofie Symoens,† Delfien Syx,† Fransiska Malfait, Bert Callewaert, Julie De Backer, Olivier Vanakker, Paul Coucke, and Anne De Paepe*

Center for Medical Genetics, Ghent University Hospital, Ghent, Belgium

Communicated by Reed E. Pyeritz

Received 9 March 2012; accepted revised manuscript 1 June 2012.
Published online 13 June 2012 in Wiley Online Library (www.wiley.com/humanmutation).DOI: 10.1002/humu.22137

ABSTRACT: Type V collagen mutations are associated with classic Ehlers–Danlos Syndrome (EDS), but it is unknown for which proportion they account and to what extent other genes are involved. We analyzed *COL5A1* and *COL5A2* in 126 patients with a diagnosis or suspicion of classic EDS. In 93 patients, a type V collagen defect was found, of which 73 were *COL5A1* mutations, 13 were *COL5A2* mutations and seven were *COL5A1* null-alleles with mutation unknown. The majority of the 73 *COL5A1* mutations generated a *COL5A1* null-allele, whereas one-third were structural mutations, scattered throughout *COL5A1*. All *COL5A2* mutations were structural mutations. Reduced availability of type V collagen appeared to be the major disease-causing mechanism, besides other intra- and extracellular contributing factors. All type V collagen defects were identified within a group of 102 patients fulfilling all major clinical Villefranche criteria, that is, skin hyperextensibility, dystrophic scarring and joint hypermobility. No *COL5A1/COL5A2* mutation was detected in 24 patients who displayed skin and joint hyperextensibility but lacked dystrophic scarring. Overall, over 90% of patients fulfilling all major Villefranche criteria for classic EDS were shown to harbor a type V collagen defect, which indicates that this is the major—if not only—cause of classic EDS.
Hum Mutat 33:1485–1493, 2012. © 2012 Wiley Periodicals, Inc.

KEY WORDS: type V collagen; classic Ehlers–Danlos Syndrome; EDS; *COL5A1*; *COL5A2*

Introduction

The Ehlers–Danlos syndrome (EDS) is a heritable connective tissue disorder characterized by hyperextensible skin, hypermobile joints, and generalized connective tissue fragility [Steinmann et al., 2002a]. According to the Villefranche nosology, EDS is classified in six subtypes based on clinical, biochemical, and molecular findings [Beighton et al., 1998]. For most EDS subtypes, mutations involving one of the fibrillar collagen proteins or a collagen-modifying enzyme have been identified. The major diagnostic criteria for the classic subtype of EDS (formerly EDS type I or "gravis;" MIM# 130000 and type II or "mitis;" MIM# 130010) include hyperextensible skin, tissue fragility manifested as widened atrophic scars, and joint hypermobility [Beighton et al., 1998]. In this subtype, mutations in *COL5A1* (MIM# 120215) and *COL5A2* (MIM# 120190), encoding the α1- and α2-chain of type V collagen respectively, have been reported [De Paepe et al., 1997; Nicholls et al., 1996; Richards et al., 1998; Toriello et al., 1996; Wenstrup et al., 1996]. They are associated with typical collagen fibril abnormalities in the skin, including variability in collagen fibril diameter and presence of large collagen aggregates, known as "collagen cauliflowers" [Hausser and Anton-Lamprecht, 1994]. Type V collagen is a quantitatively minor fibrillar collagen with a wide tissue distribution. Its best known isoform is the $[\alpha1(V)]_2\alpha2(V)$ heterotrimer which coassembles with type I collagen into heterotypic type I/V collagen fibrils in the extracellular matrix (ECM). Type V collagen is considered to regulate the diameter of these fibrils by retention of its large N-propeptide domain, which projects above the surface of the collagen fibril [Birk, 2001].

Previous mutational studies of *COL5A1* and *COL5A2*, which mainly focused on *COL5A1* haploinsufficiency mutations [Schwarze et al., 2000; Wenstrup et al., 2000], estimated that defects in type V collagen are found in approximately half of the patients with classic EDS [Malfait et al., 2005], but comprehensive studies in large patient cohorts are lacking. The majority of mutations are nonsense or frameshift mutations in *COL5A1* that generate a premature termination codon (PTC) and partially or totally abolish the expression of one *COL5A1* allele (*COL5A1* null-allele), with reduced production of the type V collagen protein as a consequence [Malfait et al., 2005; Mitchell et al., 2009; Schwarze et al., 2000; Wenstrup et al., 2000]. In addition, a limited number of structural mutations have been identified, including two missense mutations in the signal peptide domain [Symoens et al., 2009], two complex splice site mutations, and one homozygous missense mutation in the highly conserved α1(V)-N-propeptide domain [Giunta et al., 2002; Symoens et al., 2009; Takahara et al., 2002], and five mutations in the C-propeptide domain including two missense, two splice site and one frameshift mutation [De Paepe et al., 1997; Malfait et al., 2005; Wenstrup et al., 1996]. Finally, a few glycine substitutions and in-frame exon skipping mutations have been reported in the triple helix-encoding

Additional Supporting Information may be found in the online version of this article.

†Both authors have equally contributed to this work.

*Correspondence to: Anne De Paepe, Center for Medical Genetics, Ghent University Hospital, De Pintelaan 185, B-9000 Ghent, Belgium. E-mail: Anne.DePaepe@ugent.be

Contract grant sponsor: Fund for Scientific Research-Flanders (G.0171.05); GOA grant (12051203); "Geneskin" consortium of the European Commission (512117); Methusalem grant (08/01M01108).

domain of either the *COL5A1* or the *COL5A2* gene [Bouma et al., 2001; Burrows et al., 1998; Malfait et al., 2005; Nicholls et al., 1996; Richards et al., 1998].

Which proportion of patients with classic EDS harbor mutations in type V collagen and to what extent other genes contribute to the phenotype is not resolved at present. Only a very limited number of laboratories offer comprehensive molecular testing for classic EDS, and molecular analysis of *COL5A1* and *COL5A2* by direct Sanger sequencing is still labor intensive and technically challenging. Mutations in a few other "connective tissue" genes such as *COL1A1* and *TNXB* have been implicated in phenotypes strongly overlapping with classic EDS, but those mutations appear to be rare and the associated phenotypes show distinct differences with that of type V collagen-associated classic EDS [Nuytinck et al., 2000; Schalkwijk et al., 2001].

To better document the phenotypic and mutational spectrum of *COL5A1* and *COL5A2* defects, we performed a genetic study of type V collagen in 126 patients with an established or suspected clinical diagnosis of classic EDS. The purpose was: (1) to determine which proportion of classic EDS is accounted for by type V collagen mutations; (2) to evaluate the diagnostic value of biochemical collagen studies in classic EDS; (3) to identify which clinical findings are most predictive for the presence of a *COL5* mutation; (4) to search for genotype–phenotype correlations; and (5) to establish guidelines for diagnostic testing for classic EDS.

Materials And Methods

Patient Selection

For this study 126 patients were included in whom the diagnosis of classic EDS was established or suspected based on the Villefranche diagnostic criteria [Beighton et al., 1998]. These patients were ascertained in our genetic center or another clinical department. In all those patients, clinical findings were carefully collected either through physical examination by one of the authors or on the basis of an extensive clinical checklist filled in by another clinical geneticist, and supplemented for the majority of them with clinical photographs. The presence of the three major criteria for classic EDS, skin hyperextensibility, presence of widened atrophic scars, and generalized joint hypermobility, was evaluated, as well as the presence of the following "minor" clinical manifestations of classic EDS, that is, smooth and velvety skin, easy bruising, molluscoid pseudotumors and/or subcutaneous spheroids, muscle hypotonia, and history of delayed gross motor development. The presence of a positive family history for classic EDS was recorded. For 121 probands, we obtained a skin biopsy, whereas for five probands only genomic DNA was available. In 21 patients, transmission electron microscopy (TEM) was previously performed on a skin biopsy.

The study was approved by the Ethics Committee of the Ghent University Hospital, Ghent, and informed consent was obtained.

Cell Culture and Biochemical Analysis of Collagen Molecules

Fibroblast cell culture, steady state collagen labeling and subsequent sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) analysis were performed as described previously [Malfait et al., 2005]. Briefly, at confluency the patients' fibroblasts were labeled with C-Proline [Nuytinck et al., 1996] and intracellular and secreted (pro)collagen proteins were separated by SDS-PAGE. The gels were processed for fluorography, dried, and exposed to an X-ray film for evaluation of the fibrillar collagens profiles.

Isolation of DNA and RNA

For the 121 patients from whom a skin biopsy was available, fibroblast cultures were established from which genomic DNA was extracted using the DNeasy kit (Qiagen, Frankfurt, Germany). For affected and unaffected family members from which a blood sample was available genomic DNA was isolated using the PureGene® DNA Purification Kit (Gentra systems, Minneapolis, MN). Total RNA was isolated from the probands' skin fibroblasts cultured in the absence or presence of the mRNA stabilizing agent cycloheximide (Sigma-Aldrich, St. Louis, MO) using TRIZOL (Life Technologies, Invitrogen, Carlsbad, CA) or the RNeasy Mini kit (Qiagen) [Bateman et al., 1999]. For the conversion to cDNA, random hexanucleotide primers and MMLV-RTase (Invitrogen) were used according to the manufacturers' instructions.

Molecular Analysis

COL5A1 null-allele assay

To evaluate if both *COL5A1* alleles were expressed, heterozygosity for four single nucleotide polymorphisms (SNPs) was examined at the genomic DNA and cDNA level as previously described [Malfait et al., 2005]. These included: c.738C>T in exon 5 [Cappa et al., 1995], c.4122G>A in exon 52, c.4482G>C or c.4482G>A in exon 58 and c.5599T>C in exon 66 [Greenspan and Pasquinelli, 1994].

Molecular analysis

Initially, SSCP (Single-Strand Conformation Polymorphism [Orita et al., 1989]), CSGE (Conformation Sensitive Gel Electrophoresis [Ganguly et al., 1993]) or denaturing High Pressure Liquid Chromatography (dHPLC) analysis on a WAVE DNA Fragment Analysis System (Transgenomics, Cheshire, UK) [Malfait et al., 2005] were used for molecular analysis of the *COL5A1* gene on cDNA and genomic DNA and of the *COL5A2* gene on cDNA. Subsequently, direct Sanger sequencing of *COL5A1* genomic and *COL5A2* cDNA was performed, respectively, using the ABI3100, ABI3130XL, or ABI3730XL automated sequencer (Applied Biosystems, Foster City, CA). Recently, *COL5A2* genomic primers were designed and optimized (primer sequences available upon request). Also, the previously used *COL5A1* genomic primers were checked for the presence of SNPs and redesigned where necessary. All patients in whom initial studies did not reveal a causal mutation were reanalyzed with the new *COL5A1*/*COL5A2* primers. In those patients with a *COL5A1* null-allele but in whom the underlying mutation remained unknown, *COL5A1* cDNA analysis was performed on total mRNA which was isolated from fibroblasts treated with cycloheximide in order to stabilize the mutant transcript. In the patients in whom cDNA analysis revealed an exon skip, but in whom no causal mutation was identified at the genomic DNA level, primer sets of the respective gene were combined to span and sequence the deleted region. To exclude the presence of multiexon deletions within *COL5A1* or *COL5A2* in mutation-negative patients, cDNA primer sets were combined separately for the *COL5A1* gene and the *COL5A2* gene to span large coding regions. Finally, genomic DNA from mutation-negative patients was screened for mutations in *TNXB* by direct Sanger sequencing (primer sequences available

upon request). When a mutation was identified, the parents and unaffected and affected family members of the proband were investigated.

Nucleotide numbering corresponds to the *COL5A1* and *COL5A2* reference sequence (NM_000093.3 and NM_000393.3, respectively) and mutation nomenclature follows the HGMD guidelines. All mutations were checked with the Mutalyzer software (https://mutalyzer.nl/batchNameChecker) and were submitted to the LOVD Ehlers–Danlos Syndrome Variant Database (http://www.le.ac.uk/genetics/collagen/). A mutation was considered to be pathogenic if one or more of the following criteria were satisfied: (1) generation of a premature termination codon (PTC), (2) a previously published mutation which was included in the LOVD EDS Variant Database [Dalgleish, 1997, 1998], (3) a glycine substitution in the Gly-X-Y repeat of the triple helix domain, (4) a substitution or deletion of a cysteine residue in the C-propeptide domain, (5) a de novo mutation in the index patient predicted to be deleterious by the Alamut software and absent in 105 control samples (Czika and Berry, 2002), (6) genomic deletions removing (part of) an exon, (7) splice site mutations with a predicted altered splicing using the Splice Site Prediction by Neural Network (http://www.fruitfly.org/seq_tools/splice.html; Reese et al., 1997) and the Human Splicing Finder, Version 2.4 (HSF: http://www.umd.be/HSF/) [Desmet et al., 2009] software. When mRNA was available mRNA studies were performed to study the outcome of a particular splice site mutation.

Results

Biochemical Analysis of Dermal (Pro)Collagen Proteins

SDS-PAGE of intracellular and secreted procollagen and collagen proteins produced by the patients' fibroblasts was done for all 121 patients from whom skin fibroblast cultures had been established (Table 1). In 12 out of 121 cell lines, a decreased intensity of the type V collagen protein bands was seen, while the pattern for types I and III (pro)collagen was normal. In all other cell lines (109/121), normal profiles were seen for type V, as well as for types I and III (pro)collagen. This allowed us to exclude the diagnosis of other EDS subtypes, such as the vascular, kyphoscoliotic, arthrochalasis and dermatosparaxis subtypes, as well as the presence of arginine-to-cysteine substitutions in the α1(I)-collagen chain which have been previously associated with a classic EDS-like phenotype [Nuytinck et al., 2000].

Transmission Electron Microscopy

For 21 patients, TEM studies were performed. In 20 patients, TEM showed a large variability in collagen fibril diameter and presence of collagen cauliflowers. Often, deposition of granulo-filamentous material was noticed in the interfibrillar spaces. Cross-sectional pictures showed distorted and twisted collagen fibrils (courtesy of Dr. I. Hausser, Heidelberg, Germany, and Dr. T. Hermanns-Lê, Liège, Belgium). In addition, in several patients a dilated endoplasmic reticulum (ER) was observed.

In one patient, TEM showed collagen fibrils with a normal uniform diameter and absence of the typical collagen cauliflowers.

COL5A1 Null-Allele Assay

A *COL5A1* null-allele assay was performed for all 121 patients from whom skin fibroblasts were available (Table 1). Loss of expression of one *COL5A1* allele was confirmed in 49 of them. In 61 probands, the presence of a *COL5A1* null-allele was excluded, since heterozygosity for at least one of the four SNPs at both the genomic and cDNA level was observed, indicating that both *COL5A1* alleles were expressed. For 11 probands, results were non-informative due to homozygosity for the applied SNPs at genomic DNA level.

Mutation Analysis of *COL5A1*, *COL5A2*, and *TNXB*

Mutation analysis was performed in all 126 patients. In 93 of them, we could show causal involvement of type V collagen. Seventy-three patients harbored a *COL5A1* mutation, 13 patients had a *COL5A2* mutation (Fig. 1) and in seven patients we could demonstrate a *COL5A1* null-allele, although the underlying mutation remained undetected (Table 1). Forty-nine of these mutations have not yet been reported before (Table 1), four of them were detected twice (p.(Arg594*), p.(Arg1133*), p.(Arg1257*), and p.(Gly1492Ser)) in this study.

Forty-eight of the 73 *COL5A1* mutations were compatible with loss-of-expression of one *COL5A1* allele (*COL5A1* null-allele, Fig. 1, Table 1) and comprised 42 patients in whom the presence of a *COL5A1* null-allele was proven by the *COL5A1* null-allele assay, four patients who had noninformative *COL5A1* null-allele results and two patients from whom only genomic DNA was available. The identified mutations included 17 nonsense mutations, 12 small genomic deletions, 10 small genomic duplications, and 9 mRNA splicing errors.

Twenty-three of the 73 *COL5A1* mutations were "structural" mutations scattered throughout the gene, affecting different domains of the α1(V)-chain (Fig. 1, Table 1). They comprised 15 missense mutations, five mRNA splicing mutations, and three in-frame genomic deletions. The 15 missense mutations included two leucine substitutions in the α1(V)-signal peptide, two cysteine substitutions in the α1(V)-C-propeptide domain, and 11 glycine substitutions, respectively, three in the α1(V)-N-propeptide, seven in the α1(V)-triple helix domain, and one in the α1(V)-C-propeptide domain. Five patients harbored an mRNA splicing mutation of which four generated a complex splicing outcome and one activated a cryptic splice site within the affected exon. In these five patients, the synthesis of mutant in-frame transcripts was demonstrated. Two patients carried an in-frame deletion of a single exon, which was caused by a large genomic deletion encompassing the respective exon and parts of the adjacent intronic sequences. Finally, in one patient a small in-frame genomic deletion (p.(Phe1766_Glu1772delinsLeu)) in the C-propeptide domain was detected, which deletes six amino acids preceding the last two cysteine residues involved in intrachain disulfide bonding.

Two patients were found to carry a *COL5A1* mRNA splicing mutation in the consensus donor- or acceptor-splice site (+1 or –1 intronic position), of which the outcome could not be determined due to growth arrest of the fibroblasts (Fig. 1).

Of the 13 mutations in *COL5A2* (Fig. 1), eight were shown to cause in-frame skipping of a single exon, three were glycine-to-arginine substitutions, and one involved a small genomic in-frame deletion in the triple helix-encoding domain. Furthermore, we identified one single-nucleotide deletion (p.(Ile1433Thrfs*43)) in the C-propeptide, which causes the introduction of a PTC in the last *COL5A2* exon, known to escape nonsense-mediated mRNA decay (Table 1). The latter generates a mutant α-chain in which the last two cysteine residues involved in intrachain disulfide bonding are removed but replaced by a new cysteine residue.

Table 1. Overview of the Identified *COL5A1* and *COL5A2* Mutations

Pat nr	Exon	Dom	B	Change at nucleotide level	Change at protein level	Mutation Type	NA	Reference
COL5A1								
COL5A1_00050	1	SP	N	c.74T>G	p.(Leu25Arg)	**Missense**	–	[Malfait et al., 2005]–[Symoens et al., 2009]
COL5A1_00051	1	SP	N	c.74T>C	p.(Leu25Pro)	**Missense**	–	[Symoens et al., 2009]
AN_001901	2	N-pro	N	c.160G>T	p.(Gly54*)	Nonsense	+	
AN_001902	3	N-pro	N	c.379C>T	p.(Gln127*)	Nonsense	+	
COL5A1_00022	3	N-pro	N	c.466delC	p.(Arg156Glyfs*24)	Out-of-frame del	+	[Malfait et al., 2005]
COL5A1_00029	IVS3	N-pro	N	c.491+2T>G	p.(Ser94_Lys164del)	Splice out-of-frame	+	[Malfait et al., 2005]
AN_001903	4	N-pro	N	c.554dupA	p.(Lys186Glufs*29)	Out-of-frame dup	+	
COL5A1_00048	IVS4	N-pro	N	c.655-2A>G	p.[Gly219_Tyr262del; Gly219_Glu338del]	**Splice in-frame**	–	[Takahara et al., 2002]
COL5A1_00016	5	N-pro	N	c.664C>T	p.(Gln222*)	Nonsense	+	[Malfait et al., 2005]
AN_001904	IVS6	N-pro	N	c.925-1G>C	p.[Tyr263_Glu338del; Tyr263_Asn388del]	**Splice in-frame**	–	
COL5A1_00099	IVS6	N-pro	N	c.925-2A>G	p.[Tyr263_Glu338del; Tyr263_Asn388del]	**Splice in-frame**	–	[Symoens et al., 2011]
AN_001905	5	N-pro	N	c.701_702dupAT	p.(Asp235Metfs*53)	Out-of-frame dup	+	
AN_001906	7	N-pro	N	c.1053_1063delGTATGATGACC	p.(Tyr352Hisfs*44)	Out-of-frame del	NP	
AN_001907	8	N-pro	↓	c.1293delG	p.(Pro432Argfs*126)	Out-of-frame del	+	
AN_001908	10	N-pro	N	c.1402G>T	p.(Glu468*)	Nonsense	+	#
AN_001909	10	N-pro	N	c.1418delC	p.(Pro473Glnfs*85)	Out-of-frame del	+	
AN_001910	10	N-pro	N	c.1424G>A	p.(Gly475Asp)	**Missense**	–	
AN_001911	11	N-pro	N	c.1477G>A	p.(Gly493Arg)	**Missense**	–	
AN_001912	12	N-pro	N	c.1502delC	p.(Pro501Leufs*57)	Out-of-frame del	+	
COL5A1_00026	13	N-pro	↓	c.1588G>A homoz	p.(Gly530Ser)	**Missense**	–	[Giunta et al., 2002]
COL5A1_00018	16	Helix	↓	c.1780C>T	p.(Arg594*)	Nonsense	+	[Malfait et al., 2005]
COL5A1_00018	16	Helix	N	c.1780C>T	p.(Arg594*)	Nonsense	+	[Malfait et al., 2005]
COL5A1_00014	17	Helix	N	c.1855G>T	p.(Gly619*)	Nonsense	+	[Malfait et al., 2005]
AN_001913	18	Helix	N	c.1906delG	p.(Ala636Leufs*168)	Out-of-frame del	+	
AN_001914	20	Helix	N	c.2014delA	p.(Arg672Glyfs*132)	Out-of-frame del	+	
AN_001915	23	Helix	N	c.2159dupC	p.(Gly721Argfs*5)	Out-of-frame dup	+	
COL5A1_00040	27	Helix	N	c.2374C>T	p.(Arg792*)	Nonsense	ni	[Wenstrup et al., 2000]
AN_001916	IVS27	Helix	N	c.2385+1G>A		Splice site	+	
AN_001917	28	Helix	N	c.2385+263_2430+908del	p.(Gly796_Lys810del)	**In-frame del**	–	#
AN_001918	28	Helix	N	c.2389delG	p.(Ala797Profs*7)	Out-of-frame del	+	
COL5A1_00038	IVS28	Helix	N	c.2430+1G>A		Splice site	ni	[Wenstrup et al., 2000]
COL5A1_00020	IVS32	Helix	N	c.2700+1G>T		Splice site	+	[Malfait et al., 2005]
AN_001919	34	Helix	N	c.2770dupC	p.(Arg924Profs*16)	Out-of-frame dup	+	
AN_001920	IVS36	Helix	N	c.2899-1G>A	p.(Gly967Alafs*107)	Splice site	ni	
AN_001921	38	Helix	↓	c.2988dupC	p.(Gly997Argfs*17)	Out-of-frame dup	+	
AN_001922	39	Helix	↓	c.3037G>T	p.(Glu1013*)	Nonsense	+	
AN_001923	39	Helix	N	c.3050dupC	p.(Gly1018Argfs*8)	Out-of-frame dup	+	
AN_001924	39	Helix	N	c.3069dupC	p.(Gly1024Argfs*2)	Out-of-frame dup	NP	
COL5A1_00024	39	Helix	N	c.3110delC	p.(Thr1037Argfs*37)	Out-of-frame del	+	[Malfait et al., 2005]
COL5A1_00028	40	Helix	N	c.3184C>T	p.(Arg1062*)	Nonsense	+	[Malfait et al., 2005]
AN_001925	41	Helix	N	c.3215G>A	p.(Gly1072Glu)	**Missense**	–	
AN_001926	42	Helix	N	c.3258+33_3366+689delins115	p.(Gly1087_Pro1122del)	**Complex in-frame**	–	[De Paepe et al., 1997]
COL5A1_00015	42	Helix	↓	c.3325_3334delCCCCAGGGAC	p.(Pro1112Argfs*161)	Out-of-frame del	+	[Malfait et al., 2005]
AN_001927	43	Helix	N	c.3397C>T	p.(Arg1133*)	Nonsense	+	
AN_001928	43	Helix	N	c.3397C>T	p.(Arg1133*)	Nonsense	+	

(Continued)

Table 1. Continued.

Pat nr	Exon	Dom	B	Change at nucleotide level	Change at protein level	Mutation Type	NA	Reference
COL5A1_00027	43	Helix	N	c.3450dupT	p.(Pro1151Serfs*8)	Out-of-frame dup	+	[Malfait et al., 2005]
AN_001929	44	Helix	N	c.3484G>A	p.(Gly1162Arg)	**Missense**	NP	
AN_001930	48	Helix	N	c.3769C>T	p.(Arg1257*)	Nonsense	+	#
AN_001931	48	Helix	N	c.3769C>T	p.(Arg1257*)	Nonsense	+	#
COL5A1_00001	49	Helix	N	c.3874G>A	p.(Glu1292Thrfs*29)	Splice out-of-frame	+	[Mitchell et al., 2009]
AN_001932	49	Helix	N	c.3877C>T	p.(Glu1292Thrfs*29)	Splice out-of-frame	+	
AN_001933	IVS49	Helix	N	c.3907-2A>G	p.(Gly1303Lysfs*181)	Splice site	ni	
AN_001934	51	Helix	N	c.4050dupC	p.(Gly1351Argfs*14)	Out-of-frame dup	+	#
AN_001935	51	Helix	N	c.4068G>T	p.(Gly1339_Ala1356del)	**Splice in-frame**	ni	
COL5A1_00023	IVS52	Helix	N	c.4123-1G>A		Splice site	+	[Malfait et al., 2005]
AN_001936	IVS54	Helix	N	c.4231-1G>A		Splice site	ni	
COL5A1_00013	55	Helix	N	c.4234G>T	p.(Glu1412*)	Nonsense	+	[Malfait et al., 2005]
COL5A1_00019	55	Helix	N	c.4291_4292delCC	p.(Pro1431Trpfs*50)	Out-of-frame del	+	[Malfait et al., 2005]
COL5A1_00012	IVS55	Helix	N	c.4339-1G>A		Splice site	+	[Malfait et al., 2005]
COL5A1_00017	56	Helix	N	c.4383delC	p.(Gly1462Alafs*26)	Out-of-frame del	+	[Malfait et al., 2005]
COL5A1_00025	58	Helix	↓	c.4456G>T	p.(Gly1486Cys)	**Missense**	–	[Malfait et al., 2005]
COL5A1_00002	58	Helix	N	c.4465G>A	p.(Gly1489Arg)	**Missense**	–	[Mitchell et al., 2009]
AN_001937	58	Helix	↓	c.4474G>A	p.(Gly1492Ser)	**Missense**	–	
AN_001938	58	Helix	↓	c.4474G>A	p.(Gly1492Ser)	**Missense**	–	
AN_001939	58	Helix	N	c.4552C>T	p.(Gln1518*)	Nonsense	ni	
COL5A1_00103	61	Helix	N	c.4691G>A	p.(Gly1564Asp)	**Missense**	NP	[de Leeuw et al., 2012]
AN_001940	62	C-pro	N	c.4720C>T	p.(Gln1574*)	Nonsense	+	
COL5A1_00042	62	C-pro	↓	c.4916G>C	p.(Cys1639Ser)	**Missense**	–	[De Paepe et al., 1997]
AN_001941	62	C-pro	N	c.4916G>A	p.(Cys1639Tyr)	**Missense**	–	
AN_001942	63	C-pro	N	c.5031dupG	p.(Ser1678Valfs*7)	Out-of-frame-dup	+	
COL5A1_00043	IVS64	C-pro	N	c.5137-11T>A		**Splice in-frame**	–	[De Paepe et al., 1997]
AN_001943	65	C-pro	N	c.5298_5315del CCTGGGCTCCAACGACGA	p.(Phe1766_Glu1772delinsLeu)	**In-frame del**	–	
AN_001944	66	C-pro	N	c.5494G>A	p.(Gly1832Arg)	**Missense**	ni	
COL5A2								
AN_001945	IVS15	Helix	N	c.1006-2A>G	p.(Gly336_Pro353del)	**Splice in-frame**	–	
AN_001946	IVS16	Helix	N	c.1059+2 T>A	p.(Gly336_Pro353del)	**Splice in-frame**	–	
AN_001947	19	Helix	N	c.1186G>C	p.(Gly396Arg)	**Missense**	–	
AN_001948	21	Helix	N	c.1401G>A	p.(Gly435_Pro467del)	**Splice in-frame**	–	
COL5A2_00019	24	Helix	N	c.1617+4A>G	p.(Gly522_Lys539del)	**Splice in-frame**	–	[Malfait et al., 2005]
AN_001949	29	Helix	N	c.1933G>A	p.(Gly645Arg)	**Missense**	–	#
AN_001950	29	Helix	N	c.1947A>G	p.(Gly642_Pro659del)	**Splice in-frame**	–	
COL5A2_00016	38	Helix	N	c.2553+2delT	p.(Gly834_Gln851del)	**Splice in-frame**	–	[Malfait et al., 2005]
AN_001951	44	Helix	N	c.3040-2A>G	p.(Gly1014_Glu1049del)	**Splice in-frame**	–	
AN_001952	44	Helix	N	c.3104_3109delGGCCCC	p.(Gly1035_Pro1037delinsAla)	**In-frame del**	–	
COL5A2_00017	45	Helix	N	c.3148-2A>G	p.(Gly1050_Arg1067del)	**Splice in-frame**	–	[Malfait et al., 2005]
AN_001953	50	Helix	↓	c.3625G>A	p.(Gly1209Arg)	**Missense**	–	
COL5A2_00014	53	C-pro	N	c.4298delT	p.(Ile1433Thrfs*43)	**Out-of-frame del**	–	[Malfait et al., 2005]

Pat nr, patient numbers correspond to the identifiers found in the LOVD EDS Variant Database (http://www.le.ac.uk/ge/collagen/); Dom, protein domain; B, biochemical findings: N, normal electrophoretic pattern of types I, III, and V collagen; ↓, decreased intensity of the type V collagen α-chains; Mutation type: mutations in bold are structural mutations, leading to the production of the mutant α-chain. NA, *COL5A1* null-allele test; +, presence of *COL5A1* null-allele; –, both *COL5A1* alleles are expressed; ni, noninformative results; NP, not performed; #, patients previously reported as mutation-negative in the study of Malfait et al., 2005. Nucleotide numbering reflects cDNA with +1 as the A of the ATG initiation codon in the reference sequence (www.hgvs.org/mutnomen, *COL5A1*: NM_000093.3, *COL5A2*: NM_000393.3). The initiation codon at protein level is codon 1.

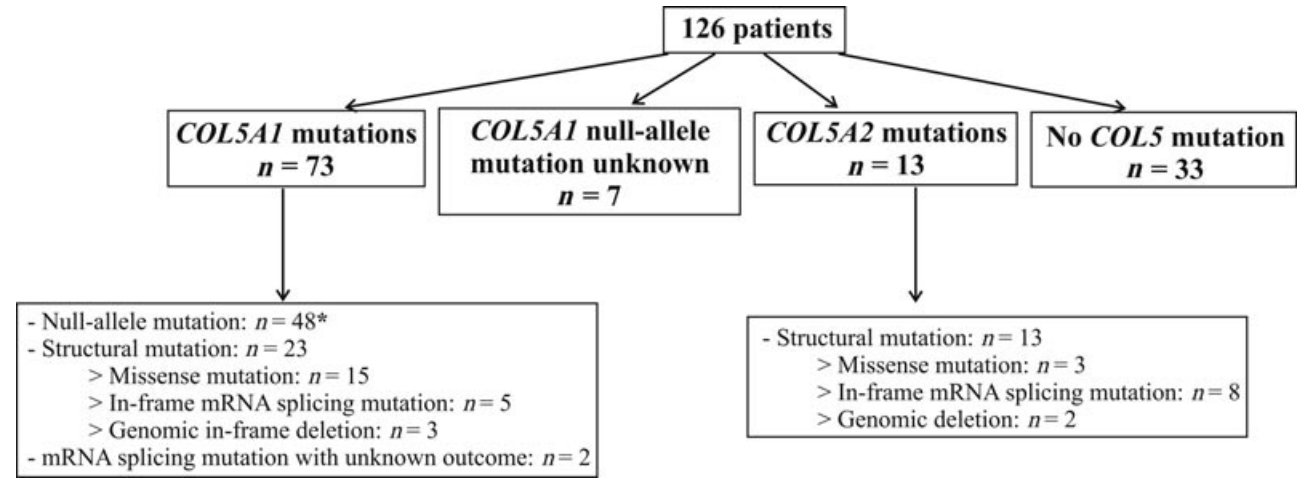

Figure 1. Flow diagram of the type V collagen defects identified in this study. The type and amount of *COL5A1* and *COL5A2* mutations is shown. ∗ Forty-eight *COL5A1* mutations leading to a *COL5A1* null-allele comprised 42 patients with a positive *COL5A1* null-allele test, four patients with a noninformative *COL5A1* null-allele test and two patients from whom only gDNA was available.

In 33 patients, no causal *COL5A1*/*COL5A2* mutation could be detected. In 27 of them, a *COL5A1* null-allele was excluded, while in six of them homozygosity for the whole *COL5A1* genomic sequence did not allow to confirm or exclude the presence of a *COL5A1* null-allele.

Furthermore, in none of the mutation-negative patients a mutation in *TNXB* could be identified.

Comparison of the biochemical results with the mutation data showed that 12 patients, in whom biochemical studies revealed abnormal results for type V collagen (Table 1), carried either a *COL5A1* null-allele ($n = 6$, five with and one without a detectable mutation) or a *COL5A1* ($n = 5$) or *COL5A2* ($n = 1$) structural mutation (Supp. Fig. S1). As such, no correlation could be found between the abnormal electrophoretic pattern for type V collagen and the mutation type. Comparison of the TEM data with the molecular data showed that all 20 patients with an abnormal TEM result harbored a type V collagen mutation. In contrast, in the patient with normal TEM no type V collagen mutation was identified.

In addition, as some authors consider heterozygosity for the p.(Gly530Ser) to be disease modifying [Giunta et al., 2002], we checked this variant in our patient population. Ten patients were heterozygous for this variant of whom six also carried a causal *COL5A1* mutation, one had a *COL5A1* null-allele without identified mutation and in three no type V collagen mutation was identified. The frequency of the heterozygous p.(Gly530Ser) substitution (in this study 8%) is in agreement with findings of previous studies [Malfait et al., 2005; Mitchell et al., 2009] and with the frequency observed in the normal population. This variant is so far the only glycine substitution in fibrillar collagens for which a clear-cut pathogenic effect has not been shown.

Clinical Evaluation and Genotype–Phenotype Correlations

Among our study cohort of 126 patients, 102 probands fulfilled all three major Villefranche criteria, that is, a smooth, velvety and hyperextensible skin, skin fragility with splitting of the skin, slow wound healing and formation of widened, atrophic scars (Fig. 2A) and hypermobility of the small and large joints (patient group 1). In addition, they presented also a combination of other manifestations of classic EDS (minor Villefranche criteria), especially easy bruising, molluscoid pseudotumors, subcutaneous spheroids, inguinal or umbilical hernia, muscle hypotonia, delayed gross motor development, and joint dislocations. In most of them, hyperlaxity of the finger joints with swan neck deformities was particularly striking (Fig. 2B). Congenital and/or progressive scoliosis was documented in 20 of them. Other clinical symptoms which were frequently present in this patient group included epicanthic folds of the eyes and protruding ears, broad and flat feet, club feet, congenital hip dislocation, and chronic joint pain. Of these 102 patients, 93 harbored a type V collagen defect, either a *COL5A1* mutation ($n = 73$), or a *COL5A2* mutation ($n = 13$), or presented a *COL5A1* null-allele without identified mutation ($n = 7$). In nine of the 102 patients, no mutation could be identified, although they clinically did not differ from the mutation-positive group. One of them carried the heterozygous p.(Gly530Ser) substitution.

Twenty-four patients of our patient population fulfilled only two major criteria of the Villefranche nosology (patient group 2), including generalized joint hypermobility, in many of them complicated by recurrent dislocations and joint pain, and a smooth or mildly hyperextensible skin, easy bruising, and sometimes mild scarring. They, however, lacked the typical doughy aspect of the skin, the skin fragility as manifested by skin splitting and dystrophic "cigarette-paper" scars over shins and elbows. None of these patients presented congenital hip dislocation, severe progressive (kypho)scoliosis, clubfeet or vascular complications. In none of these 24 patients, a type V collagen defect was identified, but two of them were heterozygous for the p.(Gly530Ser) substitution.

Forty patients of the total patient population reported a positive family history (40/126 = ∼32%). In two-thirds of the mutation-positive patients, the mutation was shown to occur de novo.

Evaluation of the phenotypic spectrum among the mutation-positive patients showed that no particular differences in the severity nor in the distribution of the clinical manifestations could be observed between patients harboring a *COL5A1* null-allele or structural mutation. Severe vascular complications were observed in two patients (patient AN_001911 - p.(Gly493Arg) in the α1(V)-N-propeptide, and patient COL5A1_00103 - p.(Gly1564Asp) in the α1(V)-triple helix domain [de Leeuw et al., 2012]) and included aneurysm and dissection of medium-sized abdominal arteries.

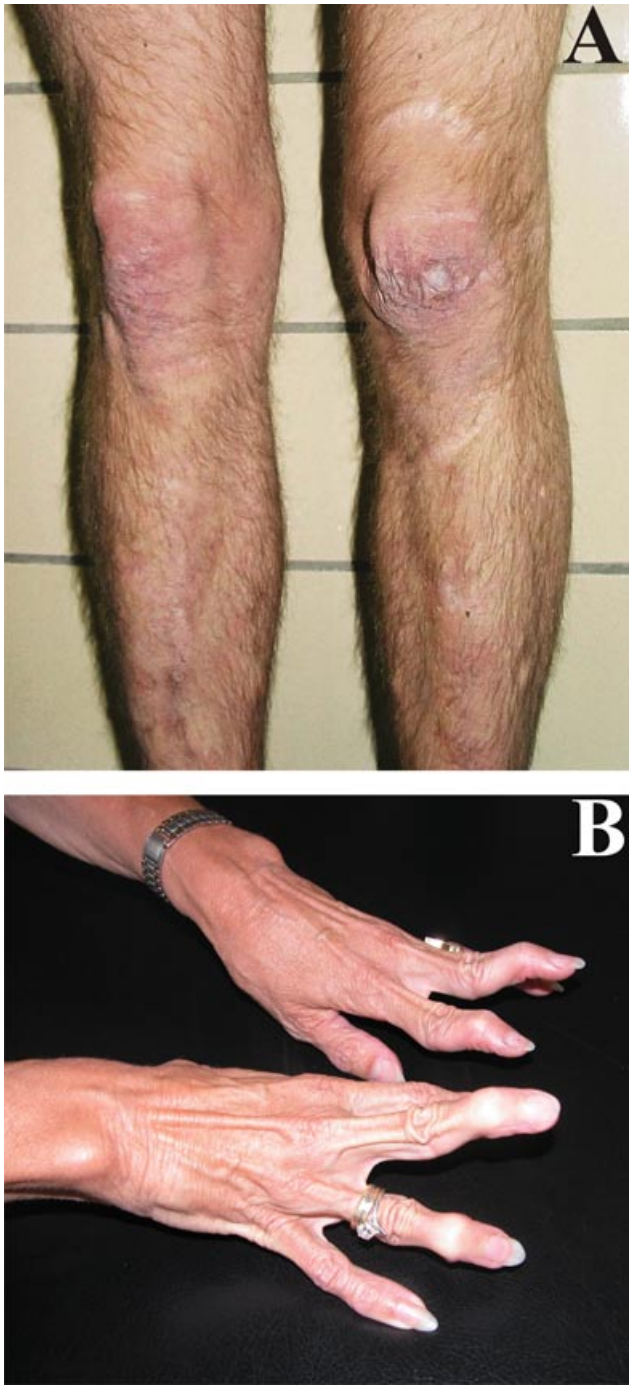

Figure 2. Clinical symptoms. **A:** Typical atrophic scars on the knees. **B:** Prominent hyperlaxity of the finger joints with swan neck deformities.

Severe or progressive cardiac-valvular disease was absent. Patients with a *COL5A2* mutation were situated within the more severe end of the phenotypic spectrum of classic EDS.

Discussion

In this study, we show that over 90% of a total of 102 patients who satisfy all three major Villefranche criteria for classic EDS (group 1) harbor a type V collagen defect, comprising 73 patients with a *COL5A1* mutation, 13 with a *COL5A2* mutation and in addition seven patients with a proven *COL5A1* null-allele but undetected mutation. In contrast, in 24 patients who present with joint hypermobility and mild skin hyperextensibility, but lack characteristic doughy and fragile skin texture with typical dystrophic scarring (group 2) no mutation could be identified. As such, our mutation data definitely implicate that *COL5A1* and *COL5A2* are the major—if not the only—genes responsible for classic EDS. This is further corroborated by the fact that exhaustive biochemical and molecular analysis failed to implicate mutations in other fibrillar collagens or in the *TNXB* gene. Although, theoretically, genetic heterogeneity cannot be excluded, we believe that technical limitations of the sequencing method may account for the inability of finding a mutation in all patients of group 1. Indeed, the mutation detection strategy applied in this study does not allow the detection of deep intronic mutations, mutations in the regulatory regions, very large genomic alterations, or whole gene deletions. Moreover, the fact that we succeeded to identify six mutations in earlier reported mutation-negative patients after revising our mutation detection method illustrates the importance of regularly updating primer sequences according to novel sequencing information which can reveal hitherto unidentified SNPs located in the primer binding site. We add 49 novel mutations to the 72 reported mutations in the LOVD EDS Variant database [Dalgleish, 1997, 1998]. Almost all are unique mutations except for four *COL5A1* mutations, which were identified twice in this study and five *COL5A1* mutations that were reported previously in other studies.

We confirm that approximately half of the type V collagen mutations are *COL5A1* haploinsufficiency mutations which generate PTC-harboring transcripts that cause rapid degradation by nonsense-mediated mRNA decay and thus decreased production of type V collagen [Malfait et al., 2005; Mitchell et al., 2009; Schwarze et al., 2000; Wenstrup et al., 2000].

In addition, 36 "structural" *COL5A1*/*COL5A2* mutations were identified of which approximately two-thirds are located in the *COL5A1* gene, and one-third in the *COL5A2* gene. Whereas *COL5A1* mutations are scattered throughout the gene, all *COL5A2* mutations are located in the triple helix domain, except one C-propeptide mutation. In agreement with previous findings, all *COL5A2* mutations identified in this study result in the production of mutant α2(V)-chains which are expected to be incorporated in the type V collagen molecules. The fact that, up to now, heterozygous *COL5A2* null-allele mutations have not been identified may suggest that this type of mutation does not result in a clinical phenotype, similar to what is observed for *COL1A2* and *COL11A1* [Marini et al., 2007; Steinmann 2002b].

The type V collagen mutation spectrum appears to be atypical compared to that of the major fibrillar collagen genes in that the number of glycine substitutions is low whereas this is the predominant mutation type in the types I and III collagen genes. Most glycine substitutions in the α1(V)-collagen chain cluster in the C-terminal part of the triple helix (exons 58 to 61). Unlike glycine substitutions, the number of splice site mutations in type V collagen does not appear to be different from what is observed for type I collagen (~20%) [Marini et al., 2007].

Whereas most *COL5A1* mutations are null-allele mutations, "structural" *COL5A1* mutations have also been shown to reduce the amount of normal type V collagen available in the ECM. We have previously shown that α1(V)-signal peptide mutations impair normal trafficking of mutant α1(V)-collagen chains through the ER [Symoens et al., 2009] and that C-propeptide mutations can prevent incorporation of the mutant proα1(V)-chains into the type V collagen heterotrimers, resulting in overall reduction of type V collagen

[De Paepe et al., 1997; Mitchell et al., 2009; Wenstrup et al., 1996]. These findings corroborate the fact that "functional" haploinsufficiency of type V collagen, caused either by loss of expression of one *COL5A1* allele, inefficient trafficking of mutant protein through the ER or impaired incorporation of mutant α-chains into type V collagen heterotrimers, is a key factor in the pathogenesis of classic EDS. In view of its important regulatory role in type I/V collagen fibrillogenesis, it is expected that reduced availability of type V collagen will negatively impact on its interactions with other ECM components and thus on ECM organisation in general. This is supported by the observation of a disorganized ECM and misassembled types I, III, and V collagen and fibronectin, as well as a disturbed localisation of their integrin receptors in a patient with a *COL5A1* C-propeptide mutation (p.(Cys1639Ser), patient COL5A1_00042) [Zoppi et al., 2004].

The exact mode of action of N-propeptide and triple helix mutations is less well understood, but it is generally accepted that these mutations result in the production of mutant α1(V)-collagen chains which participate in the formation of collagen heterotrimers, as illustrated for a *COL5A1* N-propeptide splice site mutation (c.655-2A>G) [Takahara et al., 2002]. Secreted mutant type V collagen heterotrimers may interfere with normal molecular interactions of type V collagen with other ECM constituents. In a previous study, we identified several new interaction partners for the α1(V)-N-propeptide domain, such as type VI collagen, TGF-β1 and PCPE-1 (Procollagen-C-Proteinase Enhancer 1) [Symoens et al., 2010]. This strongly suggests that the type V collagen N-propeptide plays a crucial role, not only in regulation of collagen fibrillogenesis, but also in overall ECM organisation. One of the interacting proteins, namely type VI collagen, is known to be involved in Ullrich Congenital Muscular Dystrophy (UCMD) and Bethlem myopathy. Interestingly, TEM data of skin biopsies of several UCMD patients showed the presence of some collagen cauliflowers [Kirschner et al., 2005]. Together these findings suggest that type V/type VI complexes serve as a molecular bridge in the cell-ECM environment and are essential for maintaining dermal ECM architecture.

Furthermore, inefficient ER trafficking and intracellular retention of mutant heterotrimers, as suggested by a dilated ER [Symoens et al., 2011], may decrease the amount of normal type V collagen available to the ECM and may contribute to functional haploinsufficiency of type V collagen. Accumulation of mutant type V collagen proteins in the ER may cause ER stress and activate the unfolded protein response as was shown recently for some chondrodysplasias [Boot-Handford and Briggs, 2010] and Osteogenesis imperfecta [Chessler and Byers, 1993].

In contrast to OI and type I collagen, in which *COL1A1* haploinsufficiency mutations lead to mild OI and *COL1A1*/*COL1A2* structural mutations to severe or lethal OI, no such genotype–phenotype correlation is observed for classic EDS and type V collagen. This may be related to the fact that type I collagen is much more abundant in the ECM than type V collagen and mainly has a structural "support" function, while type V collagen has a regulatory function in collagen fibrillogenesis. Our data suggest that perturbation of this regulatory function, irrespective of the nature of the type V collagen mutation, is the principal determinant of the functional and phenotypic outcome. Our findings thus suggest a common pathogenic pathway for all type V collagen mutations, in which reduced availability of normal type V collagen is a key factor, but to which other factors, such as increased ER stress and/or disturbed interactions with other ECM molecules may also contribute.

Although in most of the patients from group 1 the clinical diagnosis of classic EDS was rather straightforward, confusion with one of the other EDS subtypes can occasionally occur. For example, in some probands, the severity of the (kypho)scoliosis might suggest the possibility of kyphoscoliotic EDS. In others, especially in young children with otherwise mild skin involvement, prominent bruising or a family or personal history of a vascular incident could evoke the diagnosis of vascular EDS. In patients not fulfilling all major Villefranche criteria (group 2), the absence of prominent typical skin involvement might suggest the diagnosis of hypermobility EDS, which unlike other EDS subtypes could not be excluded with biochemical collagen studies.

Our study shows that fulfillment of all major clinical criteria for classic EDS appears to be a very reliable predictor for the presence of a type V collagen mutation. We propose to make the diagnostic Villefranche criteria for classic EDS more stringent in the way that at least all three major manifestations, that is, skin hyperextensibility, dystrophic scarring, and joint hypermobility need to be present to establish an unequivocal clinical diagnosis of classic EDS. This makes differential diagnosis with hypermobility EDS in patients with milder skin involvement more straightforward. Our data show that in case no mutation can be identified, a *COL5A1* null-allele test and TEM studies of the dermis are useful. Biochemical analysis allowed to detect an abnormality of the type V collagen protein in only approximately 10% of the patients with classic EDS, which confirms our previous observation that biochemical collagen studies are a poor diagnostic tool in this EDS subtype [Malfait et al., 2005]. Indeed, in only six of the 48 *COL5A1* null-allele mutations a decreased intensity of type V collagen was detected by biochemical collagen analysis (Supp. Fig. S1). Moreover, no correlation between the protein defect and the nature of the underlying mutation could be detected (Supp. Fig. S1). We can therefore conclude that the principal added value of the biochemical studies is to facilitate differential diagnosis with other EDS subtypes.

In conclusion, our study convincingly shows that over 90% of patients, which strictly satisfy all major Villefranche criteria for classic EDS harbor a type V collagen defect. Functional haploinsufficiency of type V collagen plays a key role in the pathogenesis of classic EDS, but other intra- and extracellular factors contribute to the disturbed collagen fibrillogenesis and disorganised ECM structure observed in classic EDS.

Acknowledgments

We thank all referring physicians for sending their patient samples to us. We are grateful to Dr. I. Hausser and Dr. T. Hermanns-Lê for performing ultrastructural studies. We thank Jozefien Weytens, Lynn Demuynck, Els De Vogelaere, Charlotte Opsomer, and Elke D'Haese for their excellent technical assistance. FM, BC, and DS are fellows of the Fund for Scientific Research (FWO). OV has a BOF research fellowship from Ghent University.

References

Bateman JF, Freddi S, Lamande SR, Byers P, Nasioulas S, Douglas J, Otway R, Kohonen-Corish M, Edkins E, Forrest S. 1999. Reliable and sensitive detection of premature termination mutations using a protein truncation test designed to overcome problems of nonsense-mediated mRNA instability. Hum Mutat 13:311–317.

Beighton P, De Paepe A, Steinmann B, Tsipouras P, Wenstrup RJ. 1998. Ehlers-Danlos syndromes: revised nosology, Villefranche, 1997. Ehlers-Danlos National Foundation (USA) and Ehlers-Danlos Support Group (UK). Am J Med Genet 77:31–37.

Birk DE. 2001. Type V collagen: heterotypic type I/V collagen interactions in the regulation of fibril assembly. Micron 32:223–237.

Boot-Handford RP, Briggs MD. 2010. The unfolded protein response and its relevance to connective tissue diseases. Cell Tissue Res 339:197–211.

Bouma P, Cabral WA, Cole WG, Marini JC. 2001. COL5A1 exon 14 splice acceptor mutation causes a functional null allele, haploinsufficiency of alpha 1(V) and abnormal heterotypic interstitial fibrils in Ehlers–Danlos syndrome II. J Biol Chem 276:13356–13364.

Burrows NP, Nicholls AC, Richards AJ, Luccarini C, Harrison JB, Yates JR, Pope FM. 1998. 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 63:390–398.

Cappa F, Caridi G, Gimelli G, Ghiggeri GM. 1995. A new biallelic DNA polymorphism of the human COL5A1 gene. Hum Genet 95:599–600.

Chessler SD, Byers PH. 1993. BiP binds type I procollagen pro alpha chains with mutations in the carboxyl-terminal propeptide synthesized by cells from patients with osteogenesis imperfecta. J Biol Chem 268:18226–18233.

Czika W, Berry JJ. 2002. Using all alleles in the multiallelic versions of the SDT and combined SDT/TDT. Am J Hum Genet 71:1235–6.

Dalgleish R. 1997. The human type I collagen mutation database. Nucleic Acids Res 25:181–187.

Dalgleish R. 1998. The Human Collagen Mutation Database 1998. Nucleic Acids Res 26:253–255.

de Leeuw K, Goorhuis JF, Tielliu IF, Symoens S, Malfait F, de Paepe A, van Tintelen JP, Hulscher JB. 2012. Superior mesenteric artery aneurysm in a 9-year-old boy with classical Ehlers–Danlos syndrome. Am J Med Genet A 158A:626–629.

De Paepe A, Nuytinck L, Hausser I, Anton-Lamprecht I, Naeyaert JM. 1997. Mutations in the COL5A1 gene are causal in the Ehlers–Danlos syndromes I and II. Am J Hum Genet 60:547–554.

Desmet FO, Hamroun D, Lalande M, Collod-Beroud G, Claustres M, Beroud C. 2009. Human Splicing Finder: an online bioinformatics tool to predict splicing signals. Nucleic Acids Res 37:e67.

Ganguly A, Rock MJ, Prockop DJ. 1993. Conformation-sensitive gel electrophoresis for rapid detection of single-base differences in double-stranded PCR products and DNA fragments: evidence for solvent-induced bends in DNA heteroduplexes. Proc Natl Acad Sci USA 90:10325–10329.

Giunta C, Nuytinck L, Raghunath M, Hausser I, De Paepe A, Steinmann B. 2002. Homozygous Gly530Ser substitution in COL5A1 causes mild classical Ehlers–Danlos syndrome. Am J Med Genet 109:284–290.

Greenspan DS, Pasquinelli AE. 1994. BstUI and DpnII RFLPs at the COL5A1 gene. Hum Mol Genet 3:385.

Hausser I, Anton-Lamprecht I. 1994. Differential ultrastructural aberrations of collagen fibrils in Ehlers–Danlos syndrome types I-IV as a means of diagnostics and classification. Hum Genet 93:394–407.

Kirschner J, Hausser I, Zou Y, Schreiber G, Christen HJ, Brown SC, Anton-Lamprecht I, Muntoni F, Hanefeld F, Bonnemann CG. 2005. Ullrich congenital muscular dystrophy: connective tissue abnormalities in the skin support overlap with Ehlers–Danlos syndromes. Am J Med Genet A 132:296–301.

Malfait F, Coucke P, Symoens S, Loeys B, Nuytinck L, De Paepe A. 2005. The molecular basis of classic Ehlers-Danlos syndrome: a comprehensive study of biochemical and molecular findings in 48 unrelated patients. Hum Mutat 25:28–37.

Marini JC, Forlino A, Cabral WA, Barnes AM, San Antonio JD, Milgrom S, Hyland JC, Korkko J, Prockop DJ, De Paepe A, Coucke P, Symoens S et al. 2007. Consortium for osteogenesis imperfecta mutations in the helical domain of type I collagen: regions rich in lethal mutations align with collagen binding sites for integrins and proteoglycans. Hum Mutat 28:209–221.

Mitchell AL, Schwarze U, Jennings JF, Byers PH. 2009. Molecular mechanisms of classical Ehlers–Danlos syndrome (EDS). Hum Mutat 30:995–1002.

Nicholls AC, Oliver JE, McCarron S, Harrison JB, Greenspan DS, Pope FM. 1996. An exon skipping mutation of a type V collagen gene (COL5A1) in Ehlers–Danlos syndrome. J Med Genet 33:940–946.

Nuytinck L, Dalgleish R, Spotila L, Renard JP, Van Regemorter N, De Paepe A. 1996. Substitution of glycine-661 by serine in the alpha1(I) and alpha2(I) chains of type I collagen results in different clinical and biochemical phenotypes. Hum Genet 97:324–329.

Nuytinck L, Freund M, Lagae L, Pierard GE, Hermanns-Le T, De Paepe A. 2000. Classical Ehlers–Danlos syndrome caused by a mutation in type I collagen. Am J Hum Genet 66:1398–402.

Orita M, Iwahana H, Kanazawa H, Hayashi K, Sekiya T. 1989. Detection of polymorphisms of human DNA by gel electrophoresis as single-strand conformation polymorphisms. Proc Natl Acad Sci USA 86:2766–2770.

Reese MG, Eeckman FH, Kulp D, Haussler D. 1997. Improved splice site detection in Genie. J Comput Biol 4:311–323.

Richards AJ, Martin S, Nicholls AC, Harrison JB, Pope FM, Burrows NP. 1998. A single base mutation in COL5A2 causes Ehlers–Danlos syndrome type II. J Med Genet 35:846–848.

Schalkwijk J, Zweers MC, Steijlen PM, Dean WB, Taylor G, van Vlijmen IM, van Haren B, Miller WL, Bristow J. 2001. A recessive form of the Ehlers–Danlos syndrome caused by tenascin-X deficiency. N Engl J Med 345:1167–1175.

Schwarze U, Atkinson M, Hoffman GG, Greenspan DS, Byers PH. 2000. 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 66:1757–1765.

Steinmann B, Royce P, Superti-Furga A. 2002a. The Ehlers–Danlos Syndrome. In: Royce P, Steinmann B, editors. Connective tissue and its heritable disorders. New York: Wiley-Liss. 431–523.

Steinmann B, Royce P, Superti-Furga A. 2002b. Chondrodysplasias: general concepts and diagnostic and management considerations. In: Royce P, Steinmann B, editor. Connective tissue and its heritable disorders. New York: Wiley-Liss, Inc. p 901–908.

Symoens S, Malfait F, Renard M, Andre J, Hausser I, Loeys B, Coucke P, De Paepe A. 2009. COL5A1 signal peptide mutations interfere with protein secretion and cause classic Ehlers–Danlos syndrome. Hum Mutat 30:E395–E403.

Symoens S, Malfait F, Vlummens P, Hermanns-Le T, Syx D, De Paepe A. 2011. A Novel Splice Variant in the N-propeptide of COL5A1 Causes an EDS Phenotype with Severe Kyphoscoliosis and Eye Involvement. PLoS One 6:e20121.

Symoens S, Renard M, Bonod Bidaud C, Syx D, Vaganay E, Malfait F, Ricard-Blum S, Kessler E, Van Laer L, Coucke P, Ruggiero F, De Paepe A. 2011. Identification of binding partners interacting with the alpha1-N-propeptide of type V collagen. Biochem J 433:371–381.

Takahara K, Schwarze U, Imamura Y, Hoffman GG, Toriello H, Smith LT, Byers PH, Greenspan DS. 2002. Order of intron removal influences multiple splice outcomes, including a two-exon skip, in a COL5A1 acceptor-site mutation that results in abnormal pro-alpha1(V) N-propeptides and Ehlers-Danlos syndrome type I. Am J Hum Genet 71:451–465.

Toriello HV, Glover TW, Takahara K, Byers PH, Miller DE, Higgins JV, Greenspan DS. 1996. A translocation interrupts the COL5A1 gene in a patient with Ehlers–Danlos syndrome and hypomelanosis of Ito. Nat Genet 13:361–35.

Wenstrup RJ, Florer JB, Willing MC, Giunta C, Steinmann B, Young F, Susic M, Cole WG. 2000. COL5A1 haploinsufficiency is a common molecular mechanism underlying the classical form of EDS. Am J Hum Genet 66:1766–76.

Wenstrup RJ, Langland GT, Willing MC, D'Souza VN, Cole WG. 1996. A splice-junction mutation in the region of COL5A1 that codes for the carboxyl propeptide of pro alpha 1(V) chains results in the gravis form of the Ehlers–Danlos syndrome (type I). Hum Mol Genet 5:1733–1736.

Zoppi N, Gardella R, De Paepe A, Barlati S, Colombi M. 2004. Human fibroblasts with mutations in COL5A1 and COL3A1 genes do not organize collagens and fibronectin in the extracellular matrix, down-regulate alpha2beta1 integrin, and recruit alphavbeta3 Instead of alpha5beta1 integrin. J Biol Chem 279:18157–18168.