A Novel Mutation in the Lysyl Hydroxylase 1 Gene Causes Decreased Lysyl Hydroxylase Activity in an Ehlers–Danlos VIA Patient

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The clinical diagnosis of a patient with the phenotype of Ehlers–Danlos syndrome type VI was confirmed biochemically by the severely diminished level of lysyl hydroxylase (LH) activity in the patient's skin fibroblasts. A novel homozygous mutation, a single base change of $T_{1360} \rightarrow G$ in exon 13 of the LH1 gene, predicted to result in W446G, was identified in the patient's full-length cDNA. This was confirmed in genomic DNA from both the patient and her parents, who were heterozygous for the mutation. This mutation was introduced into an LH1-pAcGP67 baculoviral construct and expressed, in parallel with normal LH1, in an insect cell system. The loss of LH activity in the mutated recombinant construct confirmed the pathogenicity of this mutation. Although not in the major catalytic site, this mutation occurs in a highly conserved region of the LH1 gene and may contribute to loss of activity by interfering with normal folding of the enzyme.

Key words: collagen cross-linking/collagen disorders/collagen hydroxylation/human skin fibroblasts/mutation analysis

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Patients with the rare autosomal recessive disorder Ehlers–Danlos syndrome type VIA (EDS VIA; online Mendelian inheritance in man (OMIM) no. 225400), the kyphoscoliosis type of EDS (Beighton *et al*, 1998), have the clinical phenotype of hypermobile joints, soft hyperextensible skin that is prone to bad scarring and easy bruising, and kyphoscoliosis (Byers, 2001; Steinmann *et al*, 2002). The biochemical basis for this disorder is because of severely decreased levels of lysyl hydroxylase (LH) (Pinnell *et al*, 1972), an enzyme that hydroxylates specific lysines in collagen (Kivirikko and Pihlajaniemi, 1998). The hydroxylysines are precursors for the formation of cross-links that are essential for the tensile strength of collagen. A second, rarer form of EDS VI has been described, EDS VIB (OMIM no. 229200), in which patients have the clinical phenotype of EDS VI but with normal levels of LH activity (Walker *et al*, 2004a).

In this study of a patient with the characteristic clinical phenotype of EDS VIA and severely diminished levels of LH activity, we have identified a novel homozygous mutation $T_{1360} \rightarrow G$ in exon 13 of the LH1 gene predicted to result in an amino acid change W446G. Using structure–function analysis in a baculoviral system, we have confirmed that this mutation causes the decreased LH activity associated with the clinical phenotype of EDS VIA.

Abbreviations: DHLNL, dihydroxylysinonorleucine; EDS VIA, Ehlers–Danlos syndrome type VIA; HLNL, hydroxylysinonorleucine; LH1, lysyl hydroxylase 1; OMIM, online Mendelian inheritance in man; PH, prolyl 4-hydroxylase

Patient Summary

The patient, a 5-y-old girl of Turkish origin, is the daughter of consanguineous parents. She was born at term after a normal pregnancy. At birth, she presented generalized joint laxity without obvious muscle hypotonia, and a mild scoliosis. The neuromotor development was normal in the first year of life, and the child started to walk independently at 14 mo. She then developed a progressive kyphoscoliosis causing chronic back pain. She has no history of joint dislocations or fractures. The wound healing is delayed with formation of atrophic scars. She bruises easily upon mild trauma. There is no history of internal bleeding.

Clinical examination at the age of 5 y revealed generalized joint laxity, severe kyphoscoliosis, and mild pectus excavatum (Fig 1*A* and *B*). Her skin was hyperextensible and she presented multiple atrophic scars on the legs, the hips, and the shoulders. She did not present a marfanoid habitus. Scleral hue was normal. Ultrasonography of the heart did not reveal any abnormalities. Ophthalmologic examination was not performed. The clinical investigation was conducted according to the Declaration of Helsinki principles.

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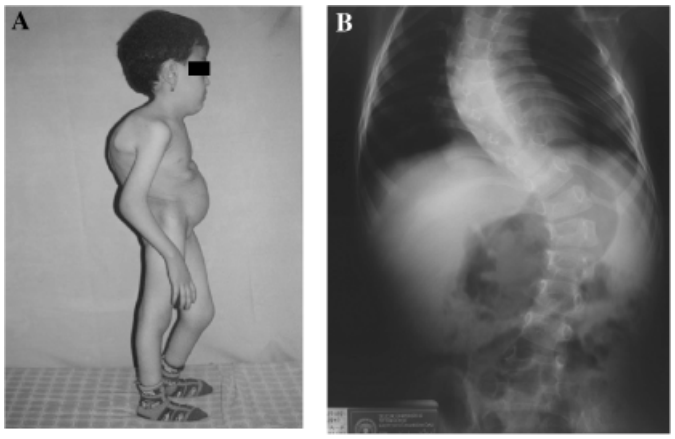


Figure 1
Patient 1270. (*A*) Patient 1270 at the age of 4 y. Note the severe kyphoscoliosis and pectus excavatum. Her skin is soft and hyperextensible. (*B*) Severe scoliosis at the age of 4 y.

Results

EDS VIA patient has severely decreased LH activity LH activity was measured in the cell extracts of skin fibroblasts cultured from the patient (1270) and a control (842), in a tritium release assay that measures predominantly helical LH activity (Walker *et al*, 2004b). The level of activity in the EDS VIA patient was significantly decreased to 17% $\pm$ 3% of control. The LH/prolyl 4-hydroxylase (PH) ratio was similarly decreased (Dembure *et al*, 1984).

Bi-functional collagen cross-linking is decreased in EDS VIA patient We then examined bi-functional collagen cross-linking in cell matrices from long-term cultures of fibroblasts from patient 1270 and three other patients characterized as EDS VIA, compared with a control. Two major reducible cross-links were identified from the analyses, i.e. dehydro-dihydroxylysinonorleucine (deH-DHLNL) and dehydro-hydroxylysinonorleucine (deH-HLNL). Those cross-links were measured as their reduced forms, DHLNL and HLNL, respectively. Other cross-links such as pyridinoline, deoxypyridinoline, and histidinohydroxylysinonorleucine were below the detectable range. As shown in Table I, the levels of the monohydroxylated cross-link HLNL did not show much variability between patients and control, whereas in patient 1270 and the three other EDS VIA patients, the levels of the dihydroxylated cross-link DHLNL and the corresponding ratios of DHLNL/HLNL were decreased to less than 20% of control.

Table I. Reducible bi-functional collagen cross-links in cells from EDS VIA patients compared with control (CON)

		EDS VIA			
	CON	**1270**	**1122**	**1268**	**1272**
DHLNL	0.52	0.09	0.1	0.08	0.07
HLNL	0.31	0.49	0.5	0.39	0.27
DHLNL/HLNL	1.7	0.19	0.2	0.2	0.27

Cross-links were measured in long-term cultures of cells as described in Materials and Methods. Reducible cross-links (moles/mole collagen) were measured as their reduced forms as shown.
DHLNL, dihydroxylysinonorleucine; HLNL, hydroxylysinonorleucine, expressed as a ratio of DHLNL/HLNL; EDS VIA, Ehlers–Danlos syndrome type VIA.

Identification of mutation in cDNA from patient 1270; confirmation of homozygosity in genomic DNA The full-length 2.2 kb cDNA for LH1 was amplified as a single transcript and directly sequenced from dermal fibroblasts from patient 1270 to identify the mutation(s) in the LH1 gene that would cause the decreased LH activity. The direct sequence analysis of the cDNA identified a single point mutation $T_{1360} \rightarrow G$ in exon 13 of the LH1 gene (TGG $\rightarrow$ GGG), which would be predicted to result in the change of a tryptophan at residue 446 to a glycine (W446G). The mutation was confirmed by PCR amplification of genomic DNA from both the patient and her parents. Direct sequencing of the 600 bp fragment covering the mutation showed that the mutation had been correctly identified and that this patient was homozygous for the mutation. The heterozygosity of both parents for this mutation confirmed the homozygosity of the mutation in their daughter.

Structure/function analysis of W446 mutation As the mutation identified in the LH1 gene from the patient, although novel, would not be predicted to either terminate or disrupt the reading frame of the LH1 gene, we utilized our baculovirus system to express the mutated LH1 and confirm that the $T_{1360} \rightarrow G$ mutation is responsible for the loss of LH activity. The $T_{1360} \rightarrow G$ mutation was introduced into the normal LH1-pAcGP67 baculoviral construct by site-directed mutagenesis, followed by cotransfection, amplification of the viral stocks, and expression in Sf9 insect cells. The non-mutated LH1cDNA baculoviral construct was amplified and expressed in parallel.

Assay of LH activity on the secreted normal and mutant proteins showed, in comparison with the high activity of normal LH1 (11.3×10^3 cpm per mg total protein), a complete loss of activity in the mutated protein. This confirmed that the predicted W446G change was responsible for the severely decreased LH activity measured in skin fibroblasts from the patient.

We used RT-PCR to examine whether degradation of LH1 mRNA in the mutant construct could be the reason for the loss of enzyme activity. In cells infected with normal and mutant LH1, however, levels of LH1 mRNA were shown to be equally expressed, indicating that mRNA degradation had not occurred (Fig 2).

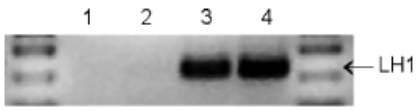


Figure 2
RT-PCR of mRNA isolated from insect cells 24 h post-infection with baculoviral stocks of recombinant normal and mutated lysyl hydroxylase 1 (LH1) RNA was isolated from insect cells and cDNA template prepared as described. One hundred fourteen base pair PCR fragments were amplified from cells infected with mutant LH1 (*lane 3*), cells infected with normal LH1 (*lane 4*), but not from cells alone (*lane 2*). Lane 1 represents the blank for the PCR reaction. LH1 mRNA appears to be equally expressed from normal and mutated LH1-baculoviral cell extracts.

Human LH1 AA #	444	445	446	447	448	449	450
Human LH1 *(Homo sapiens)*	G	V	W	N	V	P	Y
Human LH2	G	V	W	N	V	P	Y
Human LH3	G	V	W	N	V	P	Y
Chimp *(Pan troglodyte)*	G	V	W	N	V	P	Y
Pig *(Sus scrofa)*	G	V	W	N	V	P	Y
Cow *(Bos taurus)*	G	V	W	N	V	P	Y
Mouse LH1 *(Mus musculus)*	G	V	W	N	V	P	Y
Mouse LH2	G	I	W	N	V	P	Y
Mouse LH3	G	V	W	N	V	P	Y
Rat LH1 *(Rattus norvegicus)*	G	V	W	N	V	P	Y
Rat LH2	G	I	W	N	V	P	Y
Rat LH3	G	L	W	N	V	P	Y
Chicken LH1 *(Gallus gallus)*	G	P	W	N	V	P	Y
Chicken LH2	G	V	W	N	I	P	Y
Frog *(Xenopus laevis)*	G	V	W	N	V	P	Y
Zebra fish *(Tetradon nigroviridis)*	G	V	W	N	V	P	Y
Malaria mosquito *(Anopheles gambiae)*	G	L	W	N	V	P	Y
Caenorhabditis elegans	G	Y	W	N	V	P	F
Drosophila melanogaster	G	M	F	N	V	P	H

Figure 3
Alignment of the sequence region of human lysyl hydroxylase 1 (LH1) in which mutation is located with the sequence of the different human LH isoforms and the LH isoforms of different species. The location of mutation of tryptophan (W) (*black box*) in human LH1 is shown to be in a region of high homology (*gray boxes*) compared with different human LH isoforms and LH isoforms of different species.

W446G mutation is located in a highly conserved region of the LH1 gene Using NCBI's resource tools Homologene, UniGene, and BLAST (http://www.ncbi.nlm.nih.gov) and Phylo-HMM, and Multiz from the UCSC Genome Browser (http://genome.ucsc.edu), we compared the LH1 sequences in the region of the mutation between the human LH isoforms and across multiple species. As shown in Fig 3, both the tryptophan and the surrounding residues are highly conserved, suggesting that this region is important for the activity of the enzyme.

Discussion

The clinical diagnosis of EDS VIA is confirmed at the biochemical level by a deficiency of the important collagen-modifying enzyme, LH. This enzyme deficiency results from different mutations in the LH1 gene, many of which code for premature termination codons or disrupt the reading frame of LH1 (Yeowell and Walker, 2000). In this study, we have identified a novel homozygous point mutation in exon 13 of the LH1 gene that changes a codon for tryptophan to a codon for glycine at amino acid residue 446 (W446G) in a patient with EDS VIA. Although this mutation does not terminate or cause a frameshift in the LH1 gene, we have shown by structure–function analysis that it is responsible for the severely diminished levels of LH activity in this patient.

The diminished LH activity in EDS VIA patients leads to decreased hydroxylysine content in collagen. Hydroxylysine residues have two important functions. They are essential for the stability of the intermolecular collagen cross-links that provide collagen with its tensile strength, and their hydroxyl groups can serve as sites for the addition of carbohydrate units, either the monosaccharide galactose or the disaccharide glucosylgalactose (Kivirikko and Pihlajaniemi, 1998). The underglycosylation that results from the decreased hydroxylysine content of collagen in EDS VIA patients causes increased electrophoretic mobility of the α chains of type I collagen. We have observed this increased mobility of collagen chains in the current patient (data not shown), which is a consequence of the loss of LH activity and supports the diagnosis of EDS VIA. The location of the hydroxylysines within the collagen molecule, either in the telopeptide or helical domains, plays an important role in the formation of specific intermolecular collagen cross-links that determine the strength of the collagen matrix. In skin fibroblasts, the two measurable major cross-links, DHLNL and HLNL (as their reduced forms), are formed by condensation reactions between telopeptidyl hydroxylysine-aldehyde and helical hydroxylysine and between telopeptidyl lysine-aldehyde and helical hydroxylysine, respectively. A combination of telopeptidyl hydroxylysine-aldehyde and helical lysine is also possible, but in soft connective tissues the former is predominant. Therefore, if the level of the DHLNL or ratio of the DHLNL/HLNL cross-links is reduced, this may imply a decrease in telopeptide lysine hydroxylation. We observed significantly decreased DHLNL levels in long-term cultures of fibroblasts both from the patient and three additional patients with confirmed EDS VIA. Although LH1 is considered to act as a helical lysine hydroxylase, these cross-linking results indicate that this isoform also has telopeptide LH activity. This telopeptide-based activity has been demonstrated in studies showing increased levels of the tri-functional cross-link, pyridinoline, in cells expressing recombinant rat LH1 (Mercer *et al*, 2003). As this cross-link is formed via the telopeptidyl hydroxylysine-aldehyde pathway, this provides further evidence for the telopeptide LH activity of LH1.

The $T_{1360} \rightarrow G$ mutation that we have characterized in LH1 in the EDS VIA patient is a novel point mutation in exon 13 of the gene predicted to code for W446G. As this mutation lacks the obviously disruptive features of, for example, introducing a premature termination codon or interfering with the reading frame of the LH1 gene, we proved that this mutation was causal for the decreased LH activity by structure–function analysis using an LH1cDNA-pAcGP67 baculoviral construct. We have previously used this system to individually mutate the ten cysteines in the LH1 gene and determine their relative contribution to LH activity (Yeowell *et al*, 2000a). In this study, we introduced the mutation into the normal LH1 baculoviral construct by site-directed mutagenesis, amplified viral stocks, and expressed both normal and mutated recombinant proteins in their potentially secretable forms. The lack of activity in the mutant LH1 secreted fraction as compared with the high activity of non-mutated LH1 indicated that W446 was required for LH activity. Moreover, this result strongly suggested that this mutation is causal for the clinical phenotype of EDS VIA in patient 1270.

During the course of our analysis of mutations in the LH1 gene in EDS VIA patients, we have often observed a lack of detectable mRNA for LH1 in northern analysis of RNA isolated from the patients's fibroblasts. This has been generally attributed to the presence of nonsense or frameshift mutations in the LH1 gene that make the mRNA subject to rapid mRNA degradation (Maquat, 1995). To examine whether the point mutation we had identified in the LH1 gene made it vulnerable to accelerated mRNA degradation, we isolated mRNA from cells in which the mutant recombinant LH1 had been expressed, together with cells expressing normal LH1.

Using RT-PCR, we showed equivalent amplification of LH1 from both the mutant and normal LH1 mRNA, indicating that this mutation permits accumulation of stable mRNA and that degradation of mRNA is not a contributing factor to the loss of activity and decreased expression of the mutant protein.

The mutations in LH1 that are causal for the clinical phenotype of EDS VIA are shown to be located throughout the LH1 gene (Yeowell and Walker, 2000). In the majority of the more than 20 different mutations identified, both homozygous and heterozygous, there is an obvious reason for why they cause such a dramatic decrease in LH activity. These include the nonsense mutations, the splice site mutations causing out-of-frame exon deletions, and the insertions and deletions that disrupt the reading frame of LH1. The W446G mutation that we have identified in this study does not have such an obvious effect. One explanation for its effect on LH activity could be because of the difference in the size of the affected amino acids. Replacement of the bulky tryptophan moiety by glycine, the smallest amino acid, could produce a significant conformational change and possibly interfere with protein folding, subsequently causing a loss of catalytic activity. We have also shown that this mutation, although not located in the highly conserved terminal region of the LH1 gene containing the catalytic site, is located in a different highly conserved region in which the tryptophan residue, with one exception, is invariant in the human LH isoforms and between species. This suggests that it may be important to evaluate this domain in future functional studies of this enzyme.

Materials and Methods

Cell culture Human dermal fibroblasts from the proband (1270), other previously described EDS VIA patients (1122, 1268, and 1272) (Yeowell *et al*, 2000b), and from a normal control (842) (GM05659, Coriell Institute for Medical Research, Camden, New Jersey) were cultured as described (Murad *et al*, 1985). Informed consent was obtained for use of these cell lines.

LH assay LH activity in extracts from fibroblasts and recombinant enzyme expressions was measured with an L-[4,5-^{3}H] lysine-labeled underhydroxylated procollagen substrate as described (Murad *et al*, 1985; Krol *et al*, 1996). PH activity was measured with a similar underhydroxylated procollagen substrate labeled with L-[4-^{3}H] proline. The enzyme activities were quantitated as released ^3H_2O in a minimum of four assays.

Electrophoretic analysis of (pro)collagen molecules Pepsin-digested procollagen isolated from patient fibroblasts labeled with ^{14}C-proline were analyzed by SDS-PAGE as described (Nuytinck *et al*, 1996).

Analysis of cross-linking in long-term fibroblast cultures Reducible bi-functional collagen cross-links were measured in long-term cultures of patients's fibroblast cell lines as previously described (Walker *et al*, 2004a).

PCR amplification and sequence analysis of DNA from patient 1270 The numbering of the primer pairs is based on LH1cDNA sequence from dermal fibroblasts (GenBank accession number M98252). All PCR reactions were performed in a Perkin-Elmer PCR Thermocycler 480 (Boston, Massachusetts). The products were sequenced at the Duke DNA sequencing facility.

Full-length LH1cDNA cDNA was prepared in a 20 μL reaction with 1 μg total RNA in ddH_2O, isolated as described (Chomczynski and Sacchi, 1987), 1 × iScript reaction mix, and 1 μL iScript Reverse Transcriptase (Bio-Rad, Hercules, California). The reaction was then incubated at 25°C (5 min); 42°C (30 min); and 85°C (5 min). This template cDNA was used to amplify full-length cDNA for LH1 using, as 5′ primer *GAT ACC TCG GCC ATG CGG CCC CT* (beginning at nt 13), and, as 3′ primer *CCA ATT AGG GAT CGA CGA CGA AGG AGA CT* (beginning at nt 2212), in a 50 μL reaction containing 0.5 μL of the prepared cDNA, 150 ng of each primer, 0.8 mM dNTP mix, 1 × *PfuUltra* HF Reaction Buffer, and 2.5 U *PfuUltra* Hotstart High-Fidelity DNA Polymerase (Stratagene, La Jolla, California). The reaction was initially denatured at 95°C (2 min) followed by three cycles of denaturation at 95°C (30 s), annealing at 73°C (30 s), and extension at 72°C (3 min). The annealing temperature was then decreased by 3°C for three cycles and maintained at 68°C (30 s) for the final 30 cycles, followed by a final extension of 72°C (10 min). Electrophoresis of the PCR products on a 1% agarose gel showed a single 2.2 kb fragment of full-length LH1cDNA. The gel-purified cDNAs were directly sequenced.

Confirmation of mutation in genomic DNA Genomic DNA was isolated from the patient's cultured skin fibroblasts using the Blood & Cell Culture DNA mini kit (Qiagen, Valencia, California). A 600 bp fragment covering the mutation was amplified by PCR between exon 12 and exon 13 using a primer pair of *GTC ATT GCC CCG CTG ATG AC* (5′ primer, beginning at nt 1231) and *GCA CAG AAG GCC ATG TCG* (3′ primer, beginning at nt 1488) in a 50 μL reaction of 2 μg of gDNA, 300 ng of each primer, and 1 × *Herculase* Hotstart PCR master mix (Stratagene). Touchdown PCR was performed as described for the cDNA amplification, beginning with an annealing temperature of 61°C (30 s) and extension at 72°C (1 min), and was maintained at 57°C (30 s) for 30 cycles with addition of 10 s per cycle to 72°C extension. Genomic DNA from the parents was similarly amplified. Electrophoresis of the PCR products on a 3% agarose gel showed predominant 600 bp PCR fragments that were gel-purified and directly sequenced.

Site-directed mutagenesis The mutation $T_{1360} \rightarrow G$ was introduced into the previously described LH1-pAcGP67 baculoviral construct (designed for the expression of LH1 in a secretable form) (Krol *et al*, 1996), so that a codon for tryptophan (TGG) was changed to a codon for glycine (GGG), using the QuikChange II Site-Directed Mutagenesis kit (Stratagene). The 11.7 kb double-stranded LH1-baculoviral DNA construct was amplified by PCR using two complementary oligonucleotide primers that introduced the desired mutation (underlined in bold as shown): 5′ primer *GG CGT GTT GGT GTC **G**GG AAT GTG CCC TAT ATT* (beginning at nt 1346) and as 3′ primer *AAT ATA GGG CAC ATT CC**C** GAC ACC AAC ACG CC* (beginning at nt 1377). Following clonal selection according to the manufacturer's protocol and DNA isolation with Qiagen's Plasmid Purification Maxi kit, the mutated LH1 constructs were sequenced to confirm successful mutagenesis and that the reading frame was correct.

Expression and analysis of mutated and normal LH1 constructs in a baculoviral system Using Sf9 insect cells in HyQ SFX-insect serum-free medium (HyClone, Logan, Utah), cotransfection, viral amplification, expression of the mutated construct in parallel with a normal construct, and assay of LH activity were carried out as described (Yeowell *et al*, 2000a).

To measure the levels of LH1 mRNA in the recombinant cellular extracts, RNA was isolated from the Sf9 insect cells 24 h post-infection with the baculoviral stocks for recombinant normal and mutant LH1, the wild-type virus, and from untreated cells, using the Qiagen RNeasy mini kit according to the manufacturer's protocol. cDNA was prepared as described and used to amplify a 114 bp PCR fragment for LH1 using as 5′ primer *CAA GCG CTC AGC TCA GTT CTT C* (beginning at nt 156) and as 3′ primer *CTT CAG CAG CCG GAC CTT CT* (beginning at nt 270), in a 50 μL reaction containing 1 μL of the prepared cDNA, 200 ng of each primer, 1.6 mM

dNTP mix, 3.5 mM $MgCl_2$, 1 × PCR Buffer II, and 2.5 U *Amplitaq Gold* DNA Polymerase (Applied Biosystems, Foster City, California). Touchdown PCR was performed with initial denaturation at 95°C (10 min), an annealing temperature of 61°C (40 s), and extension at 72°C (30 s); the annealing temperature was decreased by 2°C every two cycles for three times and was maintained at 55°C (40 s), for 30 cycles. The PCR products were separated on a 3% agarose gel with ethidium bromide and visualized under UV light.

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