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Mutational Analysis of 12 Patients with the Phenotype of Ehlers-Danlos Syndrome Type VIB Shows no Linkage to the Zinc Transporter Gene *SLC39A13*

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Keywords

Ehlers-Danlos type VIB; Zinc transporter gene *SLC39A13*; lysyl hydroxylase activity assay; connective tissue disease; polymorphisms; skin fibroblasts

To the Editor

The Ehlers-Danlos syndrome (EDS) encompasses a group of heterogeneous inherited connective tissue disorders in which patients are clinically characterized by fragile and hyperextensible skin and joint hypermobility. EDS has been divided into six distinct clinical subtypes [Beighton et al, 1998], one of which is the autosomal recessive kyphoscoliosis type (EDS VIA) (OMIM# 225400). EDS VIA is attributed to mutations in the lysyl hydroxylase 1 (*LH1*) gene (also known as *PLOD1*: procollagen-lysine, 2-oxoglutarate 5-dioxygenase) [Yeowell et al, 2000], which codes for a post-translational modifying enzyme lysyl hydroxylase (LH) important in collagen biosynthesis [Steinmann et al, 2002; Yeowell et al, 1993]. Two other enzymes in the LH family include LH2 [Valtavaara et al, 1997], which exists in alternatively-spliced forms, LH2(short) and LH2(long) [Yeowell et al, 1999], and LH3 [Passoja et al, 1998; Valtavaara et al, 1998].

A class of patients has been identified (EDS VIB) (OMIM# 229200) that has a similar clinical phenotype to EDS VIA but with normal LH activity. An earlier study on 4 EDS VIB patients showed no linkage to mutations in the genes for *LH1, 2* and *3* [Walker et al, 2004a]. A second study on 3 LH2-deficient patients, with phenotypes of mixed EDS VI and normal LH activity, showed that their deficiency of LH2 was unrelated to mutations in either the coding or proximal promoter region of the *LH2* gene [Walker et al, 2004b]. More recently, reports [Fukada et al, 2008; Giunta et al, 2008] have linked three families with EDS-like phenotypes to homozygous mutations in the zinc transporter gene *SLC39A13* (NM_152264.2). These included a homozygous 9bp in-frame deletion in exon 4 (c.483-491 del9) in 2 families [Giunta et al, 2008] and a homozygous G to A transition at nucleotide c. 221 (c.221G>A) in exon 2 predicting the non-conservative amino acid substitution G74D in *SLC39A13* in the third family [Fukada *et al*, 2008]. In addition to the typical EDS characteristics of hyperelastic, thin, bruisable skin and hypermobile joints, the patients with the 9bp deletion [Giunta et al, 2008] showed a generalized skeletal dysplasia and abnormalities of the hands, leading to the authors' description of this disorder as the "spondylocheiro dysplastic form of EDS (SCD-EDS)". Although the lysyl and prolyl

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collagen residues were underhydroxylated in these patients, their cultured skin fibroblasts showed normal enzyme activities. *In vivo*, this underhydroxylation could be attributed to an increased internal Zn^{2+} content which competed with Fe^{2+}, a cofactor required for lysyl and prolyl hydroxylation. In contrast, in the *in vitro* enzyme assay [Murad et al, 1985], addition of supplementary Fe^{2+} together with the other required cofactors resulted in normal LH and prolyl hydroxylase (PH) activities.

Based on these results, we screened 12 cell lines in our cell repository from EDS VI-like patients, with normal LH activity measured in their cultured skin fibroblasts, for mutations in this gene. These included the four EDS VIB cell lines previously reported in this journal [Walker et al, 2004a], the three EDS VI-like cell lines reported in a separate publication [Walker et al, 2004b] and five additional cell lines. The patients were selected on the basis of their general EDS VI-like clinical characteristics as described in Table I. Although not all of the clinical characteristics of this genetically heterogeneous and complex disorder have been reported in these patients, overall their clinical features strongly suggested a classification of EDS VIB. All patients were reported to have joint hypermobility and 10 patients had soft skin, with wide scarring reported in another patient. Interestingly, one patient (1266) did not manifest soft skin. Skin bruisability was reported in 6 patients and absent in one. In the five remaining patients, easily bruisable skin was not reported. No hand abnormalities were described in these patients, with the exception of 1266 who had unusually long fingers. This patient also had radioulnar synostosis. Eleven of the 12 patients were reported to have scoliosis. Two patients had aortic root dilatations, one survived an arterial rupture and mitralis insufficiency was reported in a fourth patient. Five patients were reported to have pes planus, and it was either absent or non-reported in the others. Two patients were reported to have blue sclera and dental crowding was reported in two patients. When measured, type III collagen and urinary pyridinium (Pyr) crosslinks, an alternative test for LH activity, were biochemically normal. One exception to this was in 1267, in which both Pyr and deoxy-pyridinoline (Dpyr) were increased in urine compared with controls, but the Dpyr/Pyr ratio was normal. The increased pyridinoline crosslinks may suggest high bone turnover. Fibroblasts cultured from the patients' skin had normal LH and PH activities. Although enzyme activity was not assayed on cell line 1300, urinary Pyr crosslinks were normal in this patient showing functional LH activity.

To accomplish this mutational analysis, we amplified each of the 9 exons in the *SLC39A13* gene by PCR of genomic DNA isolated from skin fibroblasts of the 12 EDS VIB cell lines, numbered as shown in Table 2. The complete exonic sequence of the *SLC39A13* gene in each cell line, together with partial intronic sequence, was aligned with the published sequence as described [Kent et al, 2002; Kent, 2002]. No loss-of-function mutations were identified in the EDS VIB cell lines. All of the base changes that we identified are reported in Table II; these include several single base changes in exons 2 and 5 and introns 1 and 2. Although sequence analysis of the gel-purified PCR fragments identified one heterozygous base change c.250A>G predicted to code for a T20A in exon 2 of cell strain 1253, no other mutation could be detected in the sequences of this cell strain to account for the EDS VIB phenotype. In exon 5 of *SLC39A13*, a silent c.765G>A was detected as either a homozygous or heterozygous base change in six out of 12 cell strains, but was not detected in cell line 1253. Sequence analysis identified three other polymorphic markers in introns 1 and 2 in the *SLC39A13* gene (intronic numbering refers to position in the human genome browser, http://genome.ucsc.edu/ [Kent et al, 2002]). These included, in intron 1, a heterozygous base change of 47388105g>a in 5 cell strains and, in intron 2, a heterozygous g>a polymorphism was detected at base 47388537 in the same 5 cell strains. A different polymorphism of 47388542c>t in intron 2 was detected as either a homozygous or heterozygous base change in seven cell strains.

Although this study in 12 cell lines has identified several polymorphic markers in the *SLC39A13* gene, it indicates that mutations in the *SLC39A13* gene do not account for the EDS VIB phenotype in these patients. This suggests that other genes may be involved in this genetically heterogeneous form of EDS VI. Recently, in several patients clinically resembling the kyphoscoliosis type of EDS VI (EDS VIA), but with normal LH activity and normal urinary pyridinoline ratios accompanied by distinct craniofacial abnormalities and joint contractures, loss-of-function mutations in the gene (*CHST14*) coding for dermatan 4-O-sulfotransferase (D4ST1) have been identified [Kosho et al, 2010; Miyake et al, 2010]. This results in the loss of dermatan sulfate of decorin proteoglycan, a key regulator of collagen fibril assembly, which may ultimately perturb collagen assembly. In addition to acting as the causative gene for an EDS VIB-like phenotype [Kosho et al, 2010; Miyake et al, 2010], mutations in the *CHST14* gene have been linked to adducted thumb-clubfoot syndrome (ATCS) [Dundar et al, 2009]. More recently, it has been suggested that EDS VIB should be described as musculocontractural EDS which, together with ATCS, represents a single clinical identity [Malfait et al, 2010]. These latest reports define the role of *CHST14* as another gene, in addition to the *SLC39A13* gene, that broadens the spectrum of EDS VIB to include ATCS and, as such, confirms the complexity of this type of collagen disorder.

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Table I

Biochemical and clinical characteristics of 12 EDS VIB patients

Cell line	1246	1247	1250	1251	1253	1254	1261	1264	1265	1266	1267	1330
LH	N	N	N	N	N	N	N	N	N	N	N	NR
PH	N	N	N	N	N	N	N	N	N	N	N	NR
Type III collagen/ urinary X-links	N/N	N/N	N/N	N/N	nr/nr	nr/nr	nr/nr	nr/nr	nr/nr	nr/nr	N/*	nr/N
Joint hypermobility	+	+	+	+	+	+	+	+	+	+	+	+
Skin hyperextensibility	nr	nr	nr	nr	nr	nr	nr	+	+	+	+	+
Soft skin	+	+	+	+	+	+	+	+	+	−	+	Wide scars
Bruisability	+	+	+	+	+	+	nr	nr	nr	nr	−	NR
Scoliosis	+	+	+	+	+	+	+	+	+	nr	+	+
Pes planus	+	+	nr	nr	nr	nr	nr	+	+	nr	−	+
Other	Arterial rupture	Aortic root dilatation, supernumary teeth	Aortic root dilatation, dental crowding	Mitralis insufficiency			OI/ EDS, deep blue sclera			Radioulnar synostosis, long fingers and toes	Short stature, blue sclera	Inguinal, umbilical hernias

Note: The numbers of each EDS VIB cell line are listed in the top row of the table. Cell lines 1246, 1247, 1250 and 1251 have been previously reported [Walker *et al*, 2004a]. Cell lines 1261 and 1266 have been reported separately [Walker *et al*, 2004b]. LH and PH represent lysyl hydroxylase and prolyl hydroxylase activities, respectively, measured in each cell line [Murad *et al*, 1985]. N: normal values; nr: not reported; minus (−): phenotype absent; plus (+): phenotype present.

* in patient 1267, abnormal urinary cross-links suggested excessive bone turnover.

Table II

Identification of single base changes in the *SLC39A13* gene in genomic DNA from 12 EDS VIB cell lines

Cell line		1246	1247	1250	1251	1253	1254	1261	1264	1265	1266	1267	1330
Exon 2	c.250 (A/A)	A/A	A/A	A/A	A/A	A/**G**	A/A	A/A	A/A	A/A	A/A	A/A	A/A
Exon 5	c.765 (G/G)	G/**A**	G/G	**A**/**A**	G/G	G/G	G/G	G/G	G/**A**	G/**A**	G/**A**	G/**A**	G/G
intron 1	*47388105 (g/g)	g/g	g/**a**	g/g	g/**a**	g/g	g/g	g/**a**	g/g	g/g	g/**a**	g/**a**	g/g
intron 2	*47388537 (g/g)	g/g	g/**a**	g/g	g/**a**	g/g	g/g	g/**a**	g/g	g/g	g/**a**	g/**a**	g/g
intron 2	*47388542 (c/c)	c/c	c/**t**	c/c	c/**t**	c/**t**	**t**/**t**	c/**t**	c/c	c/**t**	c/c	c/c	**t**/**t**

Note: The numbers of each EDS VIB cell line are listed in the top row of the table. In brief, each of the 9 exons and part of the introns in the *SLC39A13* gene were amplified by RT-PCR from genomic DNA isolated from the 12 cell lines. The DNA sequences were aligned with the published sequence [Kent *et al*, 2002] using BLAT [Kent, 2002], and single base changes were identified in exons 2 and 5 and introns 1 and 2. Identified base changes from the published sequence are highlighted in bold as either heterozygous or homozygous changes.

*intronic numbering refers to position of *SLC39A13* gene in the human genome browser: Chr11:47386627-47394623, http:/genome.ucsc.edu/ [Kent *et al*, 2002].