Homozygosity for a missense mutation in fibulin-5 (*FBLN5*) results in a severe form of cutis laxa

Bart Loeys[1], Lionel Van Maldergem[2], Geert Mortier[1], Paul Coucke[1], Sabine Gerniers[3], Jean-Marie Naeyaert[4] and Anne De Paepe[1,*]

[1]Center for Medical Genetics, Ghent University Hospital, Ghent, Belgium, [2]Centre de Genetique Humaine, Institut de Pathologie et de Genetique, Loverval, Belgium, [3]Department of Pediatrics, Sint-Lucas Hospital, Ghent, Belgium and [4]Department of Dermatology, Ghent University Hospital, Ghent, Belgium

Hereditary cutis laxa comprises a heterogeneous group of connective tissue disorders characterized by loose skin and variable systemic involvement. Autosomal dominant and recessive as well as X-linked forms have been described. Some dominant forms are caused by mutations in the elastine gene (*ELN*). The X-linked form is now classified in the group of copper transport diseases. The genetic defect underlying the autosomal recessive (AR) forms of cutis laxa is not known. The phenotypic abnormalities recently observed in a *fibulin-5* knockout mouse model are reminiscent of human AR cutis laxa type I. Both share cutis laxa, lung emphysema and arterial involvement. Molecular study of the fibulin-5 (*FBLN5*) gene in a large consanguineous Turkish family with four patients affected by AR cutis laxa type I demonstrated the presence of a homozygous missense mutation (T998C) in the *FBLN5* gene resulting in a serine-to-proline (S227P) substitution in the fourth calcium-binding epidermal growth factor-like domain of fibulin-5 protein. This amino acid substitution is predicted to have important structural and functional consequences for normal elastogenesis. As such, we provide evidence that a genetic defect in fibulin-5 (*FBLN5*, also known as *EVEC* or *DANCE*) is responsible for a recessive form of cutis laxa in humans.

INTRODUCTION

Hereditary cutis laxa refers to a heterogeneous group of connective tissue disorders characterized by cutaneous abnormalities and variable systemic manifestations. The most constant clinical feature is loose skin, sagging over the face and trunk. An autosomal dominant and two autosomal recessive forms of cutis laxa have been described. A previously defined X-linked form, caused by mutations in the *ATP7A* gene, is now classified within the group of copper deficiency syndromes and has been shown to be allelic with Menkes disease (OMIM 304150). All forms are very rare and no precise data about their prevalence are available. The autosomal dominant form (OMIM 123700), a relatively mild condition without systemic abnormalities, can be caused by mutations in the elastin gene, but molecular heterogeneity cannot be excluded (1,2). Type I autosomal recessive cutis laxa (OMIM 219100) is characterized by pulmonary emphysema, umbilical and inguinal hernias and gastrointestinal and vesicourinary tract diverticuli and has the poorest prognosis. The type II recessive form (OMIM 219200) is called cutis laxa with joint laxity and developmental

delay (3). The autosomal recessive type I is believed to be less frequent than the type II. Histopathology of skin in patients with cutis laxa reveals loss and/or fragmentation of elastic fibers (4,5). The genetic defect in the recessive forms has not yet been identified.

The role of fibulin-5 in the development of the elastic fiber network has been shown by the study of *fibulin-5*$^{-/-}$ mice (6,7). *Fibulin-5*$^{-/-}$ mice develop a marked elastinopathy owing to the disorganization of elastic fibers. These mice present after birth with loose skin, tortuosity of the arteries and emphysematous changes in the lung, a phenotype resembling cutis laxa in humans. Fibulin-5, an integrin ligand, is an extracellular matrix protein of 448 amino acids with five potential calcium-binding epidermal growth factor (EGF)-like domains. An RGD motif, a specific cell attachment sequence interacting with $\alpha v\beta 3$ and $\alpha v\beta 5$ integrins, is located in an atypical EGF domain at the N-terminus. The gene, *FBLN5*, has been mapped to human chromosome 14q31 (8). During embryogenesis, fibulin-5 is predominantly expressed in great vessels and cardiac valves. In adults, it is mainly found in tissues that contain abundant elastic fibers, including the aorta, lung, uterus and skin.

*To whom correspondence should be addressed at: Ghent University Hospital, Department of Medical Genetics, De Pintelaan 185, B-9000 Ghent, Belgium. Tel: +32 92403602; Fax: +32 92404970; Email: anne.depaepe@rug.ac.be
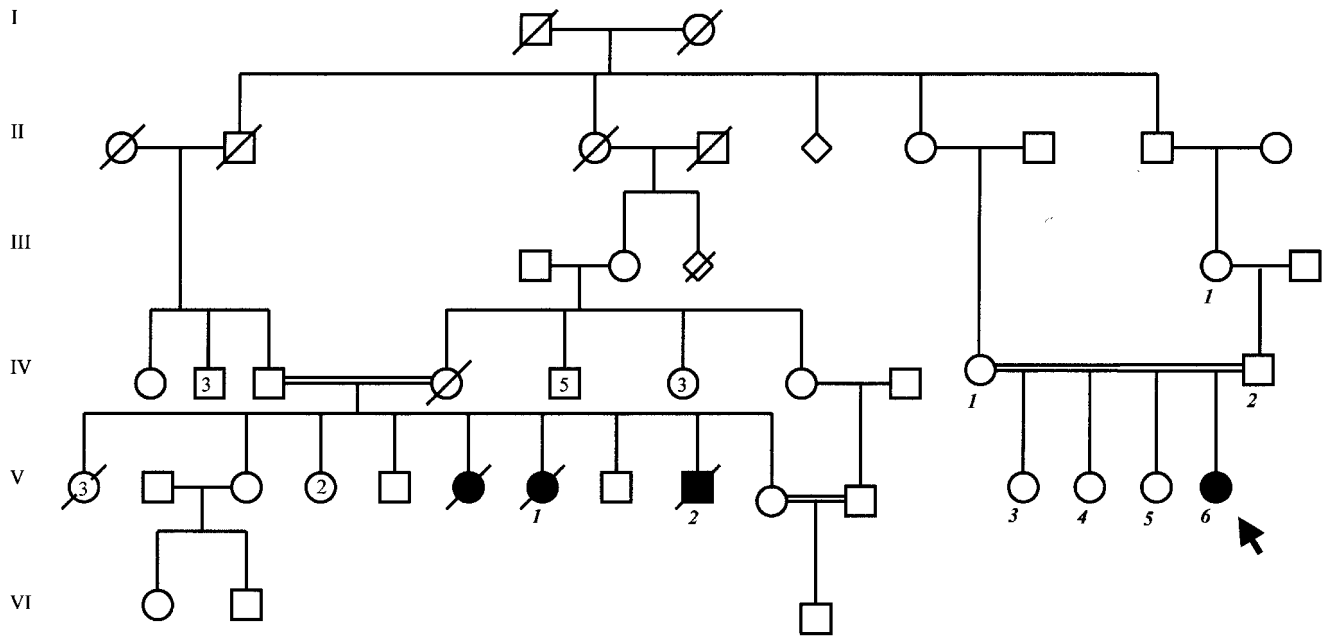
Figure 1. Pedigree of inbred kindred with autosomal recessive cutis laxa, type 1. Filled and open symbols denote clinically affected and unaffected individuals, respectively. Double horizontal line indicates consanguinity. The proband (V: 6) is indicated with an arrow. Only the individuals available for the DNA analysis are indicated by Arabic numbers (italic) beneath their respective symbols. The numbering within the symbols indicates the number of sibs represented by the symbol.

Previous studies in mice and rats have shown upregulation of fibulin-5 (EVEC or DANCE) in developing blood vessels, and in response to vascular injury such as in atherosclerosis, angioplastic restenosis and lung hyperoxia (9–11).

RESULTS

We report the identification of a homozygous missense mutation in the *FBLN5* gene in a large consanguineous Turkish family with autosomal recessive cutis laxa and pulmonary emphysema. The affected female proband (Fig. 1, individual V:6) originates from a consanguineous marriage. The parents as well as the three older sisters are in good health. The patient was born at term after an uneventful pregnancy. Birth weight was 3290 g and length was 49 cm. The diagnosis of cutis laxa was made at the age of 1 month. At that age, the loose skin was most remarkable in the face and neck, giving the baby a 'droopy' facial appearance with the impression of a downward displacement of the eyes ('sunset phenomenon') (Fig. 2A). Histologic examination of skin sections stained with van Gieson showed poorly developed elastin fibers with a marked gross granular appearance (Fig. 3A). This abnormal morphology was particularly obvious when looking at the skin histology of a normal Turkish control of the same age (Fig. 3B).

Echocardiographic evaluation showed a thickened aortic valve and supravalvular aortic stenosis (SVAS). Magnetic resonance imaging (MRI) of the brain, skeletal survey, ultrasound examination of the abdomen and ophthalmologic evaluation of the fundus did not reveal any abnormalities. On follow-up, the baby was hospitalized on several occasions because of recurrent lower respiratory tract infections and failure to thrive. At the age of 6 months, CT scan of the thorax revealed emphysema of the anterior segments of both lungs. Bronchoscopy revealed a flaccid trachea with small orificia towards the bronchi. Cytogenetic studies on blood lymphocytes showed a normal female karyotype (46, XX). The elastin gene (*ELN*) was excluded as the site of primary mutation using fluorescence *in vitro* hybridization (FISH) and linkage analysis (data not shown).

Genealogical study of the family revealed that three individuals in another branch of this large kindred presented the same cutis laxa phenotype (Fig. 2B). Three sibs, two females and one male, presented with cutis laxa, emphysema and recurrent respiratory infections. One girl also showed peripheral pulmonary artery hypoplasia leading to pulmonary hypertension, whereas the male sib had in addition recurrent renal infections associated with bladder diverticula and uretero-hydronephrosis. All sibs died following cardiorespiratory failure between age 6 months and 22 years. Their clinical and ultrastructural features have been previously reported (12).

To test our hypothesis that *FBLN5* is the disease-causing gene in this family, we performed cDNA mutation analysis of this gene in the proband. A homozygous T–C transition was identified at nucleotide position 998 of *FBLN5*. This nucleotide change is predicted to result in a serine-to-proline substitution at position 227 (S227P) in the fourth EGF-like domain of fibulin-5 (Fig. 4A), which is of the calcium-binding type (13). The other affected family members (Fig. 1, individuals V:1 and V:2), of which DNA was available, were also homozygous for this mutation. The parents of the proband (Fig. 1, individuals IV:1 and IV:2), as well as the paternal grandmother (Fig. 1, individual III:1) and one sister (Fig. 1, individual V:5), were
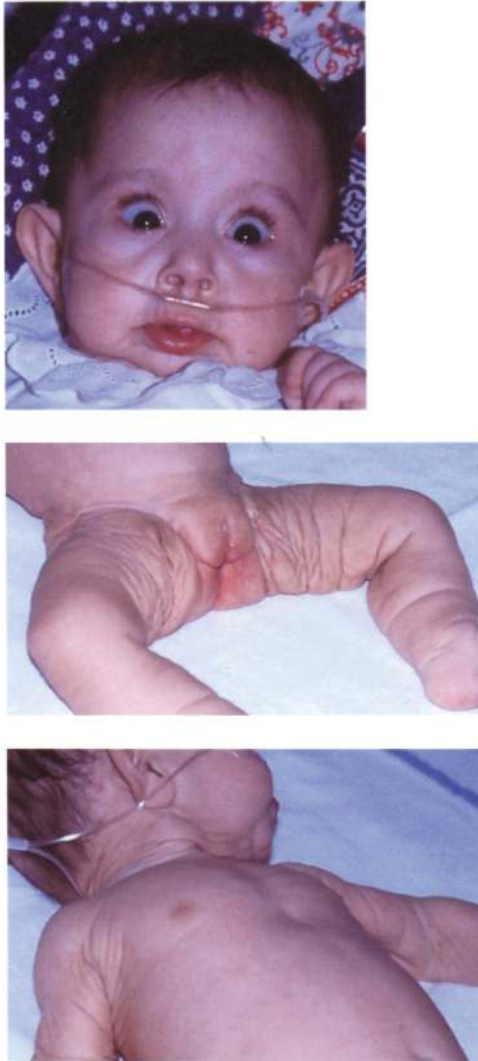
Figure 2. (A) Clinical picture of individual V:6 at age 6 months. Note sagging facial appearance, the impression of 'sunset phenomenon' and redundant skin folds over trunk and extremities. **(B)** Clinical picture of individual V:2 at birth.

heterozygous. Two other sibs (Fig. 1, individuals V:3 and V:4) were homozygous for the normal allele.

DISCUSSION

There are several arguments which indicate that the *FBLN5* mutation is responsible for the cutis laxa in this family. First, the mutation segregates with the disease phenotype. Second, the absence of the T998C mutation in a panel of 100 healthy Turkish individuals confirms that the nucleotide change is not a common polymorphism (data not shown). Third, at the protein level, Ser 227 located at the tip of the central β hairpin within the cbEGF-like domain between cysteine residues 3 and 4 is highly conserved within the fourth

cbEGF-like domain across species and in other human fibulins, such as fibulin-3 (Fig. 4B and C). Moreover, in 31 of the 43 cbEGF-like fibrillin-1 domains, the amino acid in corresponding position is a serine residue (14). Fourth, the mutation is believed to have important structural and functional consequences. This hypothesis is strengthened by the observation of highly analogous mutations in fibrillin-1 in patients with Marfan syndrome (MFS). Indeed, the very first mutation reported in MFS (R1137P) introduced a proline between the third and fourth cysteines in a cbEGF-like domain (15). It was subsequently demonstrated that R1137P alters the folding of the recombinantly expressed domain *in vitro* (16). More recently, Handford and colleagues have demonstrated that an analogous S–P change (S1953P) within the cbEGF 29 domain of fibrillin-1 leads to increased
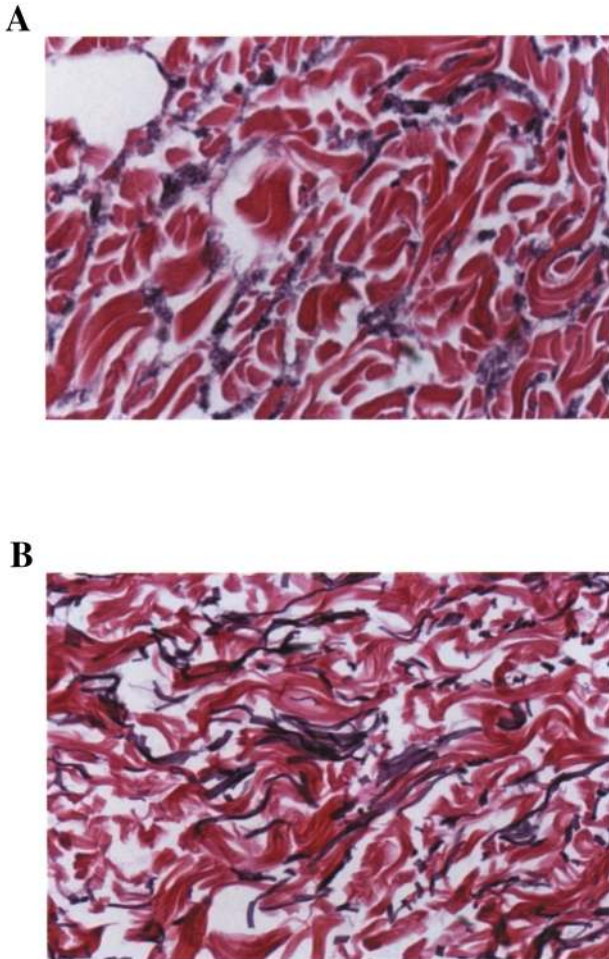
Figure 3. Skin histology (van Gieson stain): elastin fibers have a disorganized, gross granular appearance (**A**). Control skin shows well-developed elastin network with smooth individual fibers (**B**).

proteolytic susceptibility and alters the packing interactions between adjacent domains (J. Suk, P. Whiteman and P.A. Handford, manuscript in preparation). Previous studies have demonstrated that the amino acids between the third cysteine and the conserved aromatic residue between the fifth and sixth cysteines, referred to as the 'EGF core', comprise the major β sheet of the EGF fold. Proper folding of EGF-like domains has been shown to stabilize extended protein interactions, and to promote proteolytic resistance (14). In this light, S227P may contribute to disease pathogenesis by influencing the *in vivo* abundance and/or function of fibulin-5. In that the phenotype in this family associates cutis laxa and SVAS, both known elastin-deficiency states, it is likely that the quantitative and/or qualitative deficiency of fibulin-5 imposed by the S227P mutation disturbs elastogenesis. Indeed, the skin, cardiac and lung features in this family mimic those described in homozygous fibulin-5-targeted mice that show a profound defect in elastic fiber formation. Furthermore, a severe reduction of elastin and ultrastructural abnormalities of the elastic fiber network were seen in skin samples from affected patients (12).

It is notable that humans heterozygous for the S227P mutation were clinically normal. This might suggest that fibulin-5 acts as a monomer, which is in contrast to fibulin-1 and 2, which form non-covalent and disulfide-bond-linked dimers, respectively (17,18). Within the fibulin family, fibulin-5 is the first member associated with a recessive condition in humans. A mutation in fibulin-3 or *EFEMP1* (EGF-containing fibrillin-like extracellular matrix protein 1) has been shown to segregate with the autosomal dominant eye diseases Malattia Leventinese and Doney honeycomb retinal dystrophy (19). Haploinsufficiency of the *FBLN1-D* variant in a patient with disruption of *FBLN1* due to t(12;22), was associated with synpolydactyly (20). *Fibulin-1*$^{-/-}$ mice died perinatally due to massive hemorrhages and had endothelial cell abnormalities in several vessel compartments (21).

In conclusion, we have shown that a homozygous mutation in the *fibulin-5* gene is responsible for autosomal recessive cutis laxa type I and that this protein plays an important role in normal elastic fiber development in humans.

METHODS

Subjects

The family reported in this paper is of Turkish origin and was partially described previously (12). Clinical and genetic studies were carried out according to institutional guidelines and after informed consent was obtained in accordance with the Helsinki agreement. Genomic DNA and total RNA were prepared from the proband's cultured dermal fibroblasts. From all other family members, indicated with an italic number in Figure 1, only genomic DNA was available, isolated from leukocytes. Genomic DNA was extracted using the QIA-amp method (Qiagen, Inc., Valencia, CA, USA). Total RNA was obtained from the skin fibroblasts cultures with TRIzol (Invitrogen Corp., Carlsbad, CA, USA). One microgram of total RNA was used to prepare cDNA.

Mutation analysis of FBLN5

Four overlapping primer sets were designed to cover the total FBLN5 coding region (NCBI: XM_007402). These are: chFBLN5-F1, 5′-TTCTCGCCTTCGCATCTCC-3′; chFBLN5-R1, 5′-TTGGTTGCTTTCATCCATCTG-3′; chFBLN5-F2, 5′-CCACGATCTCCAGGCCTC-3′; chFBLN5-R2, 5′-TGAACGCCATCTTCCTCAAG-3′; chFBLN5-F3, 5′-ACGGCTCTTTCATCTGCCG-3′; chFBLN5-R3, 5′-GCGGGAACGGAGCGTCC-3′; chFBLN5-F4, 5′-CCCTTTACCATCTTGTACCG-3′; chFBLN5-R4, 5′-AATGAGAGGCAGCGTCGGAG-3′. After amplification, the four PCR products of the proband were cleaned by use of the QIAquick kit (Qiagen). cDNA mutation analysis of the proband was performed using direct sequencing of PCR products by means of dye terminator chemistry followed by analysis on the ABI PRISM 377 automated sequencer (Applied Biosystems, Foster City, CA, USA). Once the mutation was identified a genomic primer set (ghFBLN5-F1, 5′-AGAT-GTGAACGAGTGTGCCAC-3′; ghFBLN5-R1,
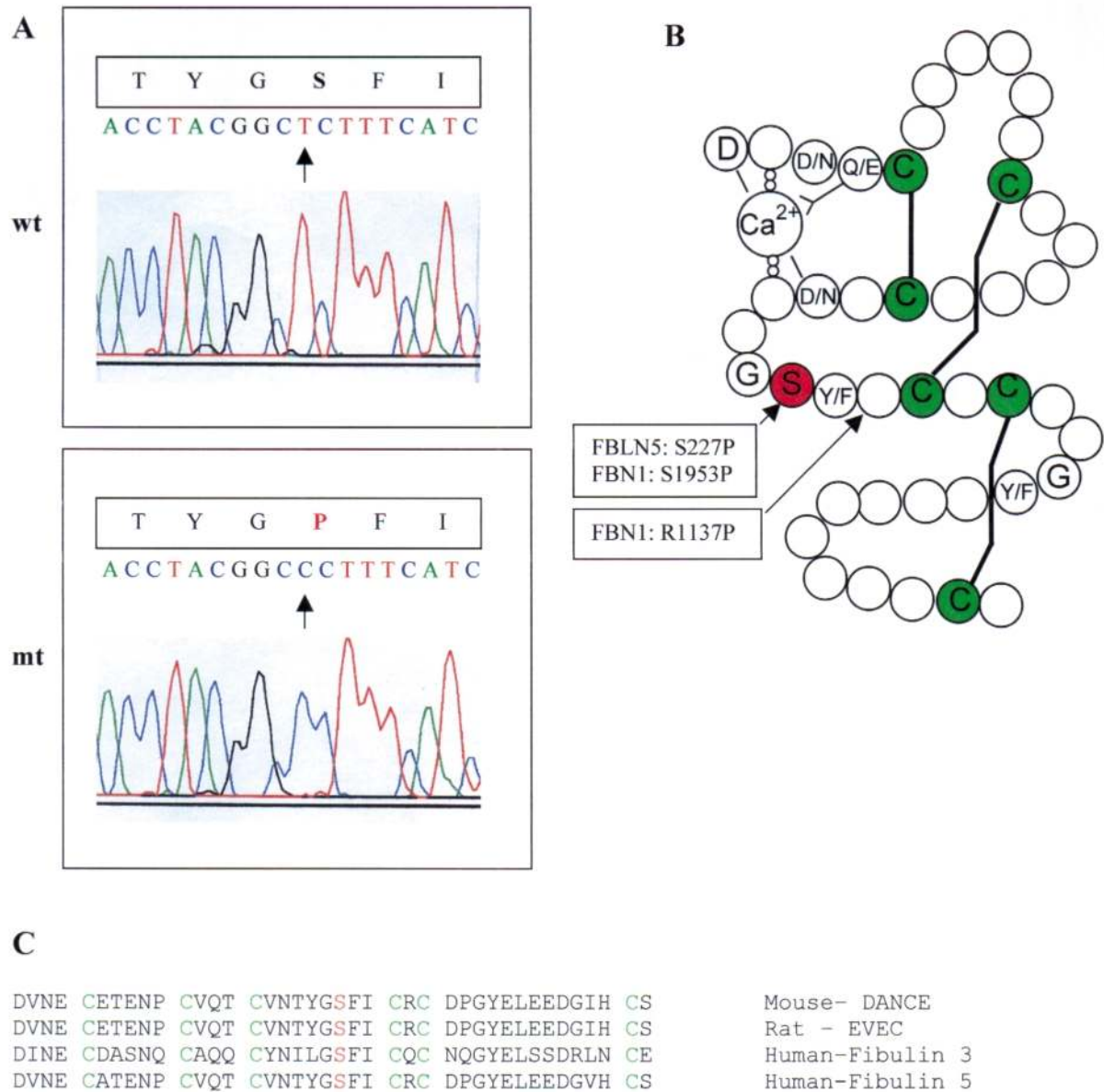
Figure 4. (A) Sequence analysis of the region flanking the T998C (S227P) mutation in *FBLN5*. The arrows point to the variant nucleotide. Sequences from control DNA (wt) and a homozygote for the mutation (mt) are shown. **(B)** Consensus sequence of cbEGF-like domain. Letters denote highly conserved amino acid residues involved in calcium binding or domain–domain packaging (14). Conserved cysteines are indicated in green. Serine, a highly conserved amino acid, is indicated in red. Positions of the mutations in FBLN5 and FBN1, as discussed in the text, are demonstrated. **(C)** Alignment of amino acid sequences from the fourth EGF-like domain of rat DANCE, mouse EVEC, and human fibulin-3 and 5, revealing the highly conserved character of the serine in position 227 of the human fibulin-5.

5′-TGAACGCCATCTTCCTCAAG-3′) flanking the mutation was designed (NCBI: BAC R-818K5 of library RPCI-11), and PCR products obtained after amplification of genomic DNA of the other family members were sequenced.

Skin biopsy

Skin biopsies were routinely processed and stained with hematoxylin, eosin and van Gieson elastin stain.

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REFERENCES

1. Zhang, M.-C., He, L., Giro, M., Yong, S.L., Tiller, G.E. and Davidson, J.M. (1999) Cutis laxa arising from frameshift mutations in exon 30 of the elastin gene (ELN). *J. Biol. Chem.*, **274**, 981–986.

2. Tassabehji, M., Metcalfe, K., Hurst, J., Ashcroft, G.S., Kielty, C., Wilmot, C., Donnai, D., Read, A.P. and Jones, C.J.P. (1998) An elastin gene mutation producing abnormal tropoelastin and abnormal elastic fibers in a patient with autosomal dominant cutis laxa. *Hum. Molec. Genet.*, **7**, 1021–1028.

3. Agha, A., Sakati, N.O., Higginbottom, M.C., Jones, K.L., Bay, C. and Nyhan, W.L. (1978) Two forms of cutis laxa presenting in the newborn period. *Acta Paediat. Scand.*, **67**, 775–780.

4. Hashimoto, K. and Kanzaki, T. (1975) Cutis laxa. Ultrastructural and biochemical studies. *Arch. Dermatol.*, **111**, 861–873.

5. Kitano, Y., Nishida, K., Okada, N., Mimaki, T. and Yabuuchi, H. (1989) Cutis laxa with ultrastructural abnormalities of elastic fiber. *J. Am. Acad. Dermatol.*, **21**, 378–380.

6. Yanagisawa, H., Davis, E.C., Starcher, B.C., Ouchi, T., Yanagisawa, M., Richardson, J.A. and Olson, E.N. (2002) Fibulin-5 is an elastin-binding protein essential for elastic fibre development in vivo. *Nature*, **415**, 168–171.

7. Nakamura, T., Lozano, P.R., Ikeda, Y., Iwanaga, Y., Hinek, A., Minamisawa, S., Cheng, C.-F., Kobuke, K., Dalton, N., Takada, Y. *et al.* (2002) Fibulin-5/DANCE is essential for elastogenesis in vivo. *Nature*, **415**, 171–175.

8. Kowal, R.C., Jolsin, J.M., Olson, E.N. and Schultz, R.A. (1999) Assignment of fibulin-5 (FBLN5) to human chromosome 14q31 by in situ hybridization and radiation hybrid mapping. *Cytogenet. Cell Genet.*, **87**, 2–3.

9. Kowal, R.C., Richardson, J.A., Miano, J.M. and Olson, E.N. (1999) EVEC, a novel epidermal growth factor-like repeat-containing protein upregulated in embryonic and diseased adult vasculature. *Circ. Res.*, **84**, 1166–1176.

10. Nakamura, T., Ruiz-Lozano, P., Lindner, V., Yabe, D., Taniwaki, M., Furukawa, Y., Kobuke, K., Tashiro, K., Lu, Z., Andon, N.L. *et al.* (1999) DANCE, a novel secreted RGD protein expressed in developing, atherosclerotic and balloon-injured arteries. *J. Biol. Chem.*, **274**, 22476–22483.

11. Jean, J.C., Eruchalu, I., Cao, Y.X. and Joyce-Brady, M. (2002) DANCE in developing and injured lung. *Am. J. Physiol. Lung Cell Mol. Physiol.*, **282**, L75–L82.

12. Van Maldergem, L., Vamos, E., Liebaers, I., Petit, P., Vandevelde, G., Simonis-Blumenfrucht, A., Bouffioux, R., Kulakowski, S., Hanquinet, S., Van Durme, P. *et al.* (1988) Severe congenital cutis laxa with pulmonary emphysema: a family with three affected sibs. *Am. J. Med. Genet.*, **31**, 455–464.

13. Handford, P.A., Mayhew, M., Baron, M., Winship, P.R., Campbell, I.D. and Brownlee, G.G. (1991) Key residues involved in calcium-binding motifs in EGF-like domains. *Nature*, **351**, 164–167.

14. Downing, A.K., Knott, V., Werner, J.M., Cardy, C.M., Campbell, I.D. and Handford, P.A. (1996) Solution structure of a pair of calcium-binding epidermal growth-like domains: implications for the Marfan syndrome and other genetic disorders. *Cell*, **85**, 597–605.

15. Dietz, H.C., Cutting, G.R., Pyeritz, R.E., Maslen, C.L., Sakai, L.Y., Corson, G.M., Puffenberger, E.G., Hamosh, A., Nanthakumar, E.J., Curristin, S.M. *et al.* (1991) Marfan syndrome caused by a recurrent de novo missense mutation in the fibrillin gene. *Nature*, **352**, 337–339.

16. Wu, Y.S., Bevilacqua, V.L. and Berg, J.M. (1995) Fibrillin domain folding and calcium binding: significance to Marfan syndrome. *Chem. Biol.*, **2**, 91–97.

17. Balbona, K., Tran, H., Godyna, S., Ingham, K.C., Strickland, D. and Argraves, W.S. (1992) Fibulin binds to itself and to the carboxyl-terminal heparin-binding region of fibronectin. *J. Biol. Chem.*, **267**, 20120–20125.

18. Sasaki, T., Wiedemann, H., Matzner, M., Chu, M.L. and Timpl, R. (1996) Expression of fibulin-2 by fibroblasts and deposition with fibronectin into a fibrillar matrix. *J. Cell Sci.*, **109**, 2895–2904.

19. Stone, E.M., Lotery, A.J., Munier, F.L., Heon, E., Piguet, B., Guymer, R.H., Vandenburgh, K., Cousin, P., Nishimura, D., Swiderski, R.E. *et al.* (1999) A single EFEMP1 mutation associated with both Malattia Leventinese and Doyne honeycomb retinal dystrophy. *Nature Genet.*, **22**, 199–202.

20. Debeer, P., Schoenmakers, E.F., Twal, W.O., Argraves, W.S., De Smet, L., Fryns, J.P. and Van De Ven, W.J. (2002) The fibulin-1 gene (FBLN1) is disrupted in a t(12; 22) associated with a complex type of synpolydactyly. *J. Med. Genet.*, **39**, 98–104.

21. Kostka, G., Giltay, R., Bloch, W., Addicks, K., Timpl, R., Fassler, R. and Chu, M.L. (2001) Perinatal lethality and endothelial cell abnormalities in several vessel compartments of fibulin-1-deficient mice. *Mol. Cell Biol.*, **21**, 7025–7034.