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ARTICLE

Altered TGFβ signaling and cardiovascular manifestations in patients with autosomal recessive cutis laxa type I caused by fibulin-4 deficiency

Marjolijn Renard[1], Tammy Holm[2], Regan Veith[3], Bert L Callewaert[1], Lesley C Adès[4], Osman Baspinar[5], Angela Pickart[3], Majed Dasouki[6], Juliane Hoyer[7], Anita Rauch[8], Pamela Trapane[9], Michael G Earing[9], Paul J Coucke[1], Lynn Y Sakai[10], Harry C Dietz[2,11], Anne M De Paepe[1] and Bart L Loeys*[,1]

Fibulin-4 is a member of the fibulin family, a group of extracellular matrix proteins prominently expressed in medial layers of large veins and arteries. Involvement of the FBLN4 gene in cardiovascular pathology was shown in a murine model and in three patients affected with cutis laxa in association with systemic involvement. To elucidate the contribution of *FBLN4* in human disease, we investigated two cohorts of patients. Direct sequencing of 17 patients with cutis laxa revealed no *FBLN4* mutations. In a second group of 22 patients presenting with arterial tortuosity, stenosis and aneurysms, *FBLN4* mutations were identified in three patients, two homozygous missense mutations (*p.Glu126Lys* and *p.Ala397Thr*) and compound heterozygosity for missense mutation *p.Glu126Val* and frameshift mutation *c.577delC*. Immunoblotting analysis showed a decreased amount of fibulin-4 protein in the fibroblast culture media of two patients, a finding sustained by diminished fibulin-4 in the extracellular matrix of the aortic wall on immunohistochemistry. pSmad2 and CTGF immunostaining of aortic and lung tissue revealed an increase in transforming growth factor (TGF)β signaling. This was confirmed by pSmad2 immunoblotting of fibroblast cultures. In conclusion, patients with recessive *FBLN4* mutations are predominantly characterized by aortic aneurysms, arterial tortuosity and stenosis. This confirms the important role of fibulin-4 in vascular elastic fiber assembly. Furthermore, we provide the first evidence for the involvement of altered TGFβ signaling in the pathogenesis of *FBLN4* mutations in humans.
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INTRODUCTION

Fibulins are a seven-member family of extracellular matrix proteins that have a role in both elastic fiber assembly and function.[1–4] Fibulin-4 is prominently expressed in the medial layers of large veins and arteries and in small capillaries.[5] The importance of fibulin-4 in elastogenesis was shown in studies of *fibulin-4*$^{-/-}$ mice that had severe lung and vascular defects including emphysema, aortic/arterial aneurysms or stenosis and arterial tortuosity.[6,7] In the aorta of the *fibulin-4*$^{-/-}$ mouse, an increase in transforming growth factor (TGF)β signaling was shown,[6] a phenomenon that has previously been described in other human aortic aneurysm syndromes, including Marfan syndrome (MFS, MIM 154700), Loeys–Dietz syndrome (LDS, MIM 610168 and 609192) and arterial tortuosity syndrome (ATS, MIM 208050).[8–10] Recently, it was shown that fibulin-4 deficiency not only leads to altered elastic fiber assembly because of its role in elastogenesis but also results in defective smooth muscle cell terminal differentiation with a downregulation of smooth muscle contractile proteins.[11] Both processes can potentially contribute, separately or in concert, to aneurysm formation.

So far, *FBLN4* (also called *EFEMP2*) mutations have been described in three patients with autosomal recessive cutis laxa type I (MIM 219100).[12–14] These patients presented prenatally with fragmented elastic tissues, cutis laxa and variable pulmonary and cardiovascular involvement (Table 1). Cardiorespiratory complications often lead to death during childhood. Mutations in *FBLN5*, the gene encoding the fibulin-5 protein, also cause autosomal recessive and autosomal dominant cutis laxa type I[15–18] and are typically associated with pulmonary emphysema. On the other hand, the X-linked form (MIM 304150), caused by mutations in the gene encoding a copper-transporting ATPase (*ATP7A*), is now classified within the group of copper-deficiency syndromes. Type II autosomal recessive cutis laxa (MIM 219200) presents with joint laxity and developmental delay. Recently, it was shown that mutations in the ATP6V0A2 gene, encoding the α2 unit of the V-type H^+ ATPase, resulted in this cutis

[1]Center for Medical Genetics, Ghent University, Ghent, Belgium; [2]McKusick-Nathans Institute of Genetic Medicine, Johns Hopkins University School of Medicine, Baltimore, MD, USA; [3]Department of Medical Genetics, Children's Hospital of Wisconsin, Milwaukee, WI, USA; [4]Department of Clinical Genetics, The Children's Hospital at Westmead, and Discipline of Paediatrics and Child Health, Sydney, Australia; [5]Department of Paediatrics, Gaziantep University, Gaziantep, Turkey; [6]Departments of Paediatrics and Internal Medicine, University of Kansas Medical Center, Kansas City, KS, USA; [7]Institute of Human Genetics, Friedrich-Alexander University of Erlangen Nuremberg, Erlangen, Germany; [8]Institute of Human Genetics, University of Zurich, Zurich-Schwerzenbach, Switzerland; [9]Department of Paediatrics, Genetics Center, Medical College of Wisconsin, Milwaukee, WI, USA; [10]Department of Biochemistry and Molecular Biology and Shriners Hospital for Children, Oregon Health and Science University, Portland, OR, USA; [11]Howard Hughes Medical Institute, Johns Hopkins University School of Medicine, Baltimore, MD, USA
*Correspondence: Dr BL Loeys, Center for Medical Genetics, University Hospital Ghent, De Pintelaan 185, Ghent B-9000, Belgium. Tel: +32 9 332 36 03; Fax: +32 9 332 49 70; E-mail bart.loeys@ugent.be
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Table 1 Overview of the clinical and molecular data of the three patients reported in this article compared with previously described patients[11–13]

	Patient 1 *Current report*	*Patient 2* *Current report*	*Patient 3* *Current report*	*Patient 4* *Dasouki et al*[12]	*Patient 5* *Hoyer et al*[14]	*Patient 6* *Hucthagowder et al*[13]
Mutation	*p.Glu126Lys* homozygous	*p.Ala397Thr* homozygous	*p.Glu126Val* *c.577delC* compound heterozygous	*p.Arg279Cys* *c.1070_1073dupCCGC* compound heterozygous	*p.Cys267Tyr* homozygous	*p.Glu57Lys* homozygous
Sex	Female	Male	Female	Female	Female	Female
Current age	20 years	7 years	Died at 18 months	Died at 27 days	Died at birth	2 years
Skin	Velvety skin normal scars	–	–	Mild cutis laxa	Generalized cutis laxa hyperextensibility	Cutis laxa
Arteries	Tortuosity aneurysm stenosis	Tortuosity aneurysm	Tortuosity aneurysm	Tortuosity aneurysm dissection	–	Tortuosity aneurysm
Emphysema	–	–	–	–	++	++
Joint laxity	–	+	–	–	–	++
Other	Arched palate retrognathia	Hypertelorism flat facies	Hypertelorism long fingers hemispheric stroke	Arachnodactyly	Hypertelorism retrognathia cardiorespiratory insufficiency	Fractures hernia diaphragmatica

laxa subtype by causing impaired glycosylation.[19] An additional form of autosomal recessive cutis laxa resembling type II is the De Barsy syndrome (MIM 219150). Patients have a progeroid-like appearance, typical facial features, growth delay and cutis laxa.[20–21] Recently, disease-causing mutations were identified in *PYCR1*, the gene encoding pyrroline-5-carboxylate reductase 1.[22–23] Patients presenting with autosomal dominant cutis laxa (MIM 123700), caused by alterations in the gene encoding the elastin protein *ELN*,[24–27] have a normal lifespan and less prominent internal organ abnormalities.

In this study we screened *FBLN4*, which encodes the fibulin-4 protein, in two phenotypically distinct cohorts, the first with cutis laxa and the second with arterial tortuosity, stenosis and aneurysms. A potential link with TGFβ signaling and the effect of the different mutations on fibulin-4 protein expression were investigated.

MATERIALS AND METHODS

Patient data

Two patient cohorts were investigated in this study. The first cohort consisted of 17 patients with predominant cutis laxa without major cardiovascular findings (Supplementary Table S1). The 22 patients in the second cohort had mild skin involvement but significant cardiovascular features, such as arterial tortuosity, stenosis and aneurysms (Supplementary Table S1). We describe here the clinical details of all three patients in whom we found *FBLN4* mutations, and summarize the clinical data of two previously reported patients in whom we studied fibroblasts or tissues.

A currently 20-year-old woman (patient 1) presented at the age of 2 months with airway compression, ascending aortic aneurysm, proximal pulmonary arterial stenosis, distal pulmonary arterial dilatation and innominate artery dilatation. She underwent cardiovascular surgery at 2.5, 7 and 8 months of age. By the age of 39 months, she had abdominal arterial tortuosity and dilatation. Clinical findings included a high arched palate, micrognathia, mild joint hypermobility, velvety skin, no cutis laxa and normal scarring. A cerebral angiogram at the age of 5 years showed arterial tortuosity of the internal carotid, anterior/middle cerebral and vertebral arteries. A histology of aortic biopsies showed disrupted elastic fibers and increased deposition of glycosaminoglycans. A detailed clinical description has been published previously.[28]

Patient 2 is a boy who was 3 years old at the time of presentation with a flat face, prominent forehead, hypertelorism, highly arched palate, pectus excavatum and joint hypermobility. He had a wide mediastinum on routine chest radiography. Echocardiography showed a dilated ascending aorta, and aortography showed pseudocoarctation and aortic tortuosity. The clinical features of this patient have been reported previously.[29] At present, the patient is 7 years old and the dilatation of his ascending aorta has increased to 54 mm. The patient is asymptomatic.

Patient 3 was born at 37 weeks of gestation. Clinical features included a prominent forehead, mild hypertelorism, downslanting palpebral fissures, depressed nasal bridge, low-set and distorted external ears and long fingers. An echocardiogram showed severe dilatation of the ascending aorta and severe tortuosity of the entire aorta with hypoplasia of the transverse aortic arch, proximal descending, thoracic and abdominal aorta. MRI showed severe tortuosity of the carotid and cerebral arteries. At 5 weeks of age, she suffered a large hemispheric stroke. She died from cardiorespiratory failure at approximately 18 months. Autopsy was declined. Neither parent of patient 3 had phenotypcial characteristics of ARCL type I.

Patient 4 was described recently.[12] This female patient presented with mild cutis laxa, arachnodactyly and systemic involvement, including pulmonary hypertension, mild tricuspid valve insufficiency, abdominal aortic tortuosity and dilatation of the ascending aorta and the main branches of the pulmonary arteries, with dissection. The parents denied consanguinity. She died at 27 days of age from respiratory distress and inoperable systemic vascular abnormalities.

Patient 5 is a child from consanguineous parents. She died shortly after birth from profound bradycardia. Clinical features included facial dysmorphism, cutis laxa, joint contractures and arachnodactyly. She had a thickened myocardium with a minimal pericardial effusion, emphysema and fragile vessels. A detailed clinical description has been reported recently.[14]

Molecular analysis

For patients 1–3, genomic DNA was extracted from EDTA blood samples using the Puregene method (Qiagen, Venlo, The Netherlands). All coding exons and flanking introns of *FBLN4* were amplified by PCR at the genomic DNA level (primer sequences are available on request). Products were sequenced using the BigDye terminator cycle sequencing method on the ABI3730 XL automatic sequencer (Applied Biosystems, Halle, Belgium).

Cell culture

Skin fibroblasts were available from patients 1 and 5, and from age- and sex-matched controls. Fibroblasts were cultured in Dulbecco's modified Eagle's medium (DMEM, Invitrogen, Merelbeke, Belgium) supplemented with 10% fetal calf serum (FCS, Invitrogen) in the presence of antibiotics at 37°C in 5% CO_2.

Protein analysis

Confluent fibroblasts of patients 1 and 5 and of age- and sex-matched controls were grown in DMEM medium in the absence of FCS for 24 h. Media were harvested and concentrated (Centriprep-30, Millipore, Brussels, Belgium) in the presence of protease inhibitors (Complete Mini tablet, Roche Applied Science, Vilvoorde, Belgium). Protein samples were loaded on gel, together with 5× nonreducing lane marker sample buffer (Thermo Fisher Scientific, Aalst, Belgium). SDS-PAGE electrophoresis was performed using NuPage 4–12% Bis-Tris precast gels (Invitrogen), followed by electrotransfer of proteins onto a Hybond-ECL nitrocellulose membrane (GE healthcare, Brussels, Belgium) using the iBlot system (Invitrogen). The membrane was blocked in 2% ECL-advantage buffer (GE healthcare). Immunoblotting was carried out using a mouse monoclonal anti-human fibulin-4 antibody (clone 226 347 597) (1:500), in combination with a mouse monoclonal anti-human fibronectin-1 antibody (clone 84) (1:500), followed by incubation with the secondary antibody ECLplex goat-anti-mouse IgG-Cy5 (1/2000) (GE healthcare). Monoclonal antibodies were generated in mice immunized with full-length recombinant human fibulin-4.[30] Epitopes for all three monoclonal antibodies are located in the N-terminal half of fibulin-4. Membranes were scanned using Typhoon 9400 (GE healthcare). Immunoblotting of pSmad2 was performed as previously described.[31] Quantification of immunoblots was performed using ImageJ software (NIH, Bethesda, MD, USA).

Histological analysis

Paraffin-embedded lung and aortic tissue samples were available from patient 4. For immunohistochemical staining of fibulin-4 (clone 347), connective tissue growth factor (CTGF) and pSmad2 (the phosphorylated form of Smad2) in lung and aortic tissue, we selected representative specimens from three healthy control individuals. From these formalin-fixed, paraffin-embedded specimens, 5 μm-thick sections were cut, deparaffinized and rehydrated. Antigens were unmasked using 10 mM sodium citrate buffer and auto-peroxidase activity was inhibited. Sections were blocked with normal goat serum (pSmad2 and CTGF) or normal horse serum (fibulin-4) (Vectastain, Burlingame, CA, USA). Antibodies directed against CTGF (Abcam, Cambridge, UK), pSmad2 (Ser465/467) (Cell Signaling Technology, Boston, MA, USA) and fibulin-4 (see protein analysis section) were used. Subsequently, sections were incubated with a secondary antibody, either goat anti-rabbit IgG (pSmad2 and CTGF) or horse anti-mouse (fibulin-4) (Vectastain), as well as with ABC (Avidin: Biotinylated enzyme Complex) reagent (Vectastain) and DAB (3,3′-Diaminobenzidine) peroxidase (Vectastain). Sections were dehydrated in xylene and mounted. Light microscopy was performed on a Zeiss Axio Imager A1 microscope (Carl Zeiss, Zaventem, Belgium).

RESULTS

FBLN4 mutation screening

We sequenced *FBLN4* in two cohorts of patients. The 17 patients of the first cohort presented predominantly with cutis laxa and had previously screened negative for *ELN* and *FBLN5* mutations. The second cohort included 22 patients who were diagnosed with arterial tortuosity, stenosis and aneurysms. This cohort screened negative previously for *SLC2A10*, *FBN1*, *TGFBR1* and *TGFBR2* mutations. Molecular study of the first cohort did not identify any *FBLN4* mutations. In the second cohort, *FBLN4* mutations were identified in three patients. Patients 1 and 2 have homozygous missense mutations, *c.376G>A* (*p.Glu126Lys*) and *c.1189G>A* (*p.Ala397Thr*) (Figure 2a and b). The first mutation replaces the same glutamic acid residue from the EGF consensus sequence as the previously reported *p.Glu57Lys*,[13] but in a different cbEGF domain, whereas the second mutation affects a highly conserved amino acid in the fibulin-type module. The parents of patient 2 were heterozygous for the *p.Ala397Thr* mutation. The third patient (Figure 1) harbors a missense mutation, *c.377A>T* (*p.Glu126Val*), in combination with a frameshift mutation, *c.577delC*, leading to a premature termination codon (*p.Gln193Ser fs X12*) (Figure 2c). Parental studies confirmed that these mutations occurred in *trans*. The missense mutation in patient 3 affects the same glutamic acid residue (at position 126) as identified in patient 1, but leads to a different amino-acid substitution. Parental samples were not available for molecular analysis. None of the mutations were identified in 200 control chromosomes. An overview of the molecular and clinical data of these three patients and of *FBLN4* mutation-positive patients described previously in the literature (patients 4–6) is given in Table 1.

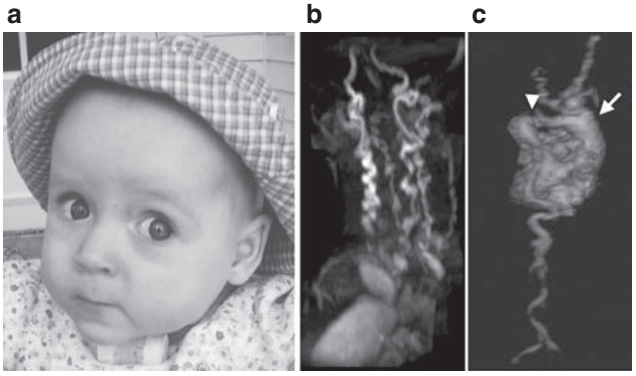

Figure 1 (**a**) Clinical photograph of patient 3 showing a prominent forehead with depressed nasal bridge, mild hypertelorism and downslanting palpebral fissures. The patient has a trachea canulae. (**b**) MRI shows severe tortuosity of the carotid arteries (anterolateral oblique view). (**c**) A posterior view of a 3D reconstruction of an MR angio showing, successively, the dilated aorta ascendens (indicated with an arrow), hypoplasia of the transverse aorta (indicated with an arrowhead) and tortuous descending aorta.

Fibulin-4 expression in human skin fibroblasts and aortic tissue

To investigate the effect of *FBLN4* mutations on the expression of fibulin-4 protein, an immunoblotting experiment was conducted on the culture medium of dermal fibroblasts of patient 1 (homozygous *c.376G>A* (*p.Glu126Lys*) missense mutation), patient 5 (homozygous *c.800G>A* (*p.Cys267Tyr*) missense mutation) and two age- and sex-matched controls. Immunoblotting showed a distinct protein band of approximately 49 kDa in the culture medium of control samples. In patient 5, a complete absence, and in patient 1, a slightly diminished amount of fibulin-4 in the culture medium were observed (Figure 3b). In addition, we performed immunohistochemical staining of fibulin-4 on aortic tissue from a control individual and from patient 4, which confirmed the near absence of extracellular fibulin-4 in the aortic wall of patient 4 in comparison with its control. Similar intracellular fibulin-4 staining was observed in both individuals (Figure 3a). These results suggest that mutant proteins are either not secreted, or are secreted into extracellular space to a lesser extent. The impaired secretion of mutant fibulin-4 leads to a decrease of available fibulin-4 protein in the extracellular matrix for elastogenesis, and disturbed elastic fiber formation.

Increased TGFβ signaling in patients with *FBLN4* mutations

To determine whether mutations in the human FBLN4 gene have an effect on the TGFβ signaling pathway, immunohistochemical staining for phosphorylated Smad2 (pSmad2, an effector of TGFβ signaling) and CTGF (a TGFβ-driven gene product) in the aorta and lungs of a normal individual and of patient 4 was performed. We demonstrated a more intense and increased nuclear pSmad2 staining in the aorta and lung of the patient, which was not present in control tissues (Figure 4). After TGFβ stimulation, immunoblotting indicated a significantly increased pSmad2 signal in fibroblast cultures of patients 1 and 5,

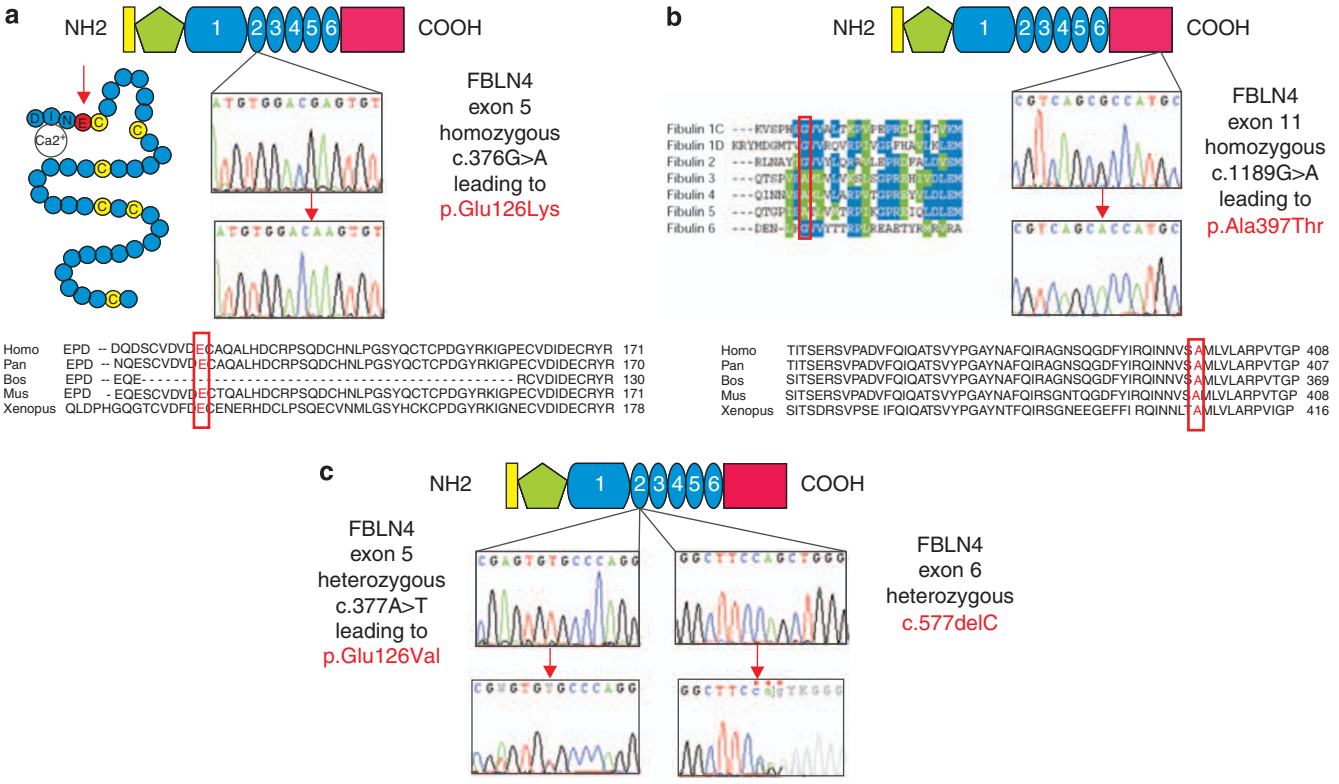

Figure 2 DNA sequence of *FBLN4* in (**a**) patient 1, showing the missense *c.376G>A* mutation, leading to *Glu126Lys* substitution in the protein. The glutamic acid residue at position 126 is evolutionarily conserved and located in the DINE consensus sequence of the second cbEGF-like domain, showing in (**b**) patient 2 the *c.1189G>A* mutation, leading to the *Ala397Thr* substitution. The alanine residue is located in the C-terminal fibulin-like module and is conserved between fibulin homologs and highly conserved among species, showing in (**c**) patient 3 the compound heterozygous *c.377A>T* missense (leading to the *Glu126Val* substitution) and *c.577delC* mutations. The missense mutation affects the same conserved glutamic acid residue as identified in patient 1 (see panel a for alignment of the conserved sequence).

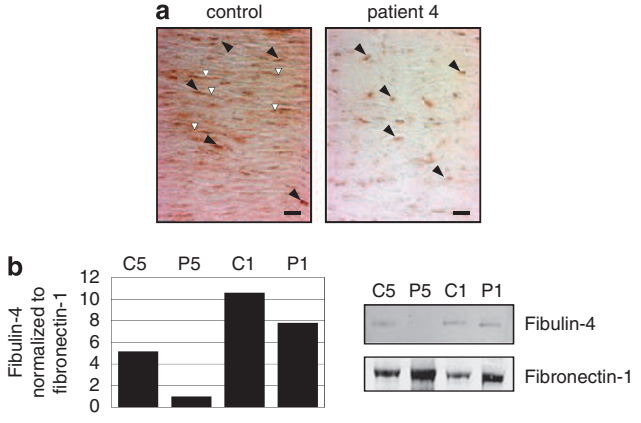

Figure 3 (**a**) Immunohistochemical staining of aortic tissue of patient 4 and its age- and sex-matched control. Intracellular fibulin-4 staining is seen in both individuals (examples of intracellular staining are indicated with a black arrow). No extracellular fibulin-4 is seen in the aorta of the patient, compared with its control (extracellular staining is indicated with white arrows). Scale bars=100 μm. (**b**) Immunoblot analysis of cultured media using fibroblasts of age- and sex-matched controls (C1 and C5) and of two patients (P1 and P5). Top panel: anti-fibulin-4 monoclonal antibody (clone 347) demonstrating impaired fibulin-4 secretion in patient 5 and slightly impaired fibulin-4 secretion in patient 1 in contrast to their matched control. Bottom panel: anti-fibronectin-1 monoclonal antibody indicating the amounts of samples loaded on the gel. The fibulin-4 secretion to the extracellular space as seen on the gel images was quantified using ImageJ software.

in comparison with their respective controls (Figure 5). These findings suggest that mutations in *FBLN4* have an effect on the TGFβ signaling pathway. This hypothesis is supported by increased intensity of staining for CTGF in the aortic and lung tissue of patient 4.

DISCUSSION

We identified *FBLN4* mutations in three unrelated patients, clinically characterized by major cardiovascular defects, including aortic aneurysms, arterial tortuosity and stenosis, and minor skin involvement. This phenotype is different from that seen in patients with mutations in the genes encoding the elastin protein, *ELN*, and the fibulin-5 protein, *FBLN5*, in which skin involvement is the most prominent clinical finding. Although the patients described in this paper share clinical characteristics with previously reported *FBLN4* mutation-positive patients,[12–14] there is considerable clinical overlap with the LDS and the ATS. LDS is an autosomal dominant disorder characterized by a triad of arterial tortuosity and aneurysms, hypertelorism and bifid uvula or cleft palate. The disorder is caused by mutations in the genes encoding TGFβ receptors 1 and 2 (*TGFBR1* and *TGFBR2*, respectively).[9,31] The autosomal recessive disorder, ATS, is characterized by tortuosity, elongation, stenosis and aneurysm formation of the major arteries.[32] Coucke *et al*[8] identified *SLC2A10*, the gene encoding a facilitative glucose transporter GLUT10, as the causative gene. The patients investigated in this study were referred because of clinical features reminiscent of one or more of these disorders. This report doubles the number of known patients with *FBLN4* mutations.[12–14] Our findings underscore the prominent vascular involvement and

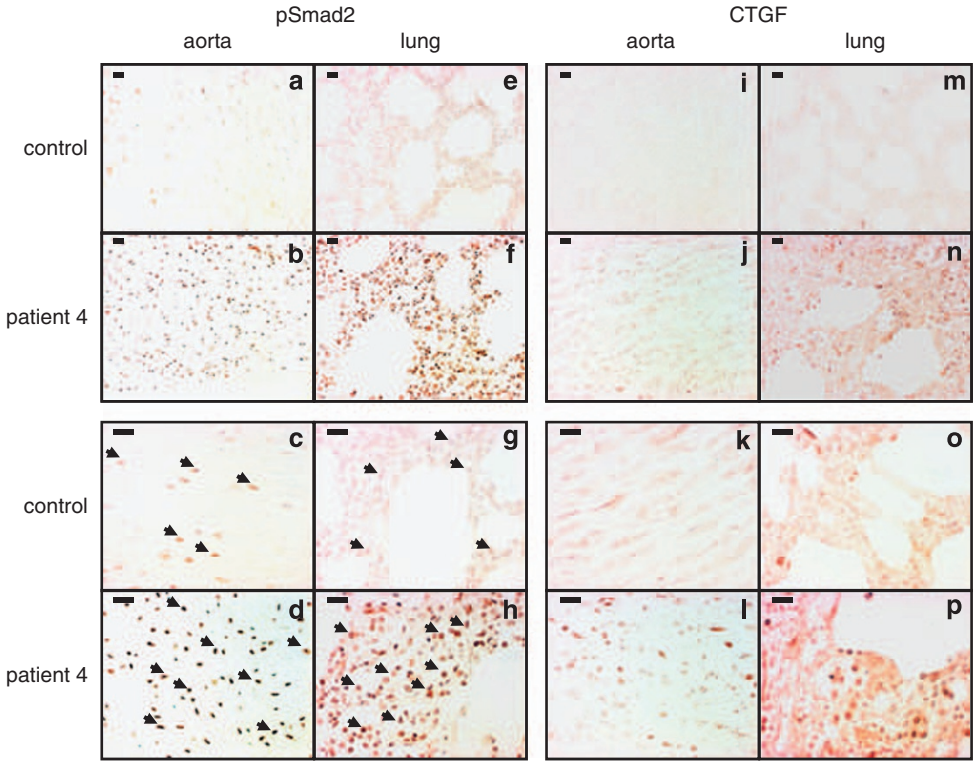

Figure 4 Immunohistochemical staining of aorta (**a**–**d** and **i**–**l**) and lung (**e**–**h** and **m**–**p**) tissue in *FBLN4* mutation-positive patient 4 (**b**, **d**, **f**, **h**, **j**, **l**, **n**, **p**) and in control (**a**, **c**, **e**, **g**, **i**, **k**, **m**, **o**). Increased nuclear accumulation of phosphorylated Smad2 is present in both the aorta (**b** ,**d**) (some of the nuclei are indicated with an arrow) and the lung (**f**, **h**) of the patient, as compared with control aorta (**a**, **c**) and lung (**e**, **g**). Similarly, increased levels of CTGF expression are noted in the aorta (**j**, **l**) and lung (**n**, **p**) of the patient, compared with control aorta (**i**, **k**) and lung (**m**, **o**). These results are indicative of increased TGFβ signaling in the *FBLN4* mutation-positive patient. Scale bars=100 μm.

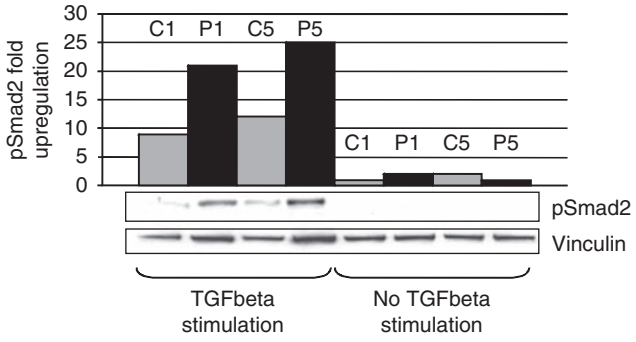

Figure 5 Immunoblot analysis of fibroblast cell extracts of age- and sex-matched controls (C1 and C5) and patients 1 (P1) and 5 (P5). The graph and the pSmad2 immunoblotting panel shows an increase in pSmad2 level in patient 1 and patient 5 when cells were stimulated with TGFβ protein, in contrast to their controls. When cells are not stimulated, basal expression of pSmad2 is seen. Bottom panel: anti-vinculin antibody, indicating the amounts of samples loaded on the gel. This immunoblotting analysis is representative of three independent experiments (see Supplementary Figure S1).

the rather mild cutaneous findings associated with *FBLN4* mutations. As such, screening of *FBLN4* should be considered particularly in patients with findings suggestive of LDS or ATS, in whom *TGFBR1*, *TGFBR2* or *SLC2A10* mutation screening is negative. We report for the first time long-term survival in patients 1 and 2, who are 20 and 7 years old, respectively. This is an interesting observation in view of the early lethality previously reported in *FBLN4* mutation-positive patients and *Fbln4*-deficient mice.[7]

We report four novel *FBLN4* mutations. Several lines of evidence suggest the disease-causing nature of these mutations. First, the three missense mutations cause either a change in charge (*p.Glu126Lys* and *p.Glu126Val*), a change of hydrophilic side chain to hydrophobic side chain (*p.Glu126Val*), or the addition of a hydroxyl group (*p.Ala397Thr*). Second, these missense mutations modify highly conserved residues within the fibulin-4 sequence. The glutamic acid residue at position 126 is located in the DINE consensus sequence of the second cbEFG-like module, which is essential for Ca^{2+} binding. Ca^{2+} binding of EGF modules is crucial for module–module interactions, and hence for the structural and binding properties of the intact protein.[33] Third, *in silico* prediction programs, Polyphen and SIFT, classify the missense mutations as probably (*p.Glu126Lys* and *p.Glu126Val*) or possibly (*p.Ala397Thr*) damaging and not tolerated, respectively. Finally, the alanine residue at position 397 is highly conserved among species and between the homologous fibulin-3, -4 and -5 proteins (Figure 2b).

Immunoblotting analysis showed the absence (patient 5) or a slightly decreased amount (patient 1) of the mutant fibulin-4 protein in the culture media of patients in comparison with controls. This result mirrors the difference in disease severity manifested clinically between these two patients. A possible explanation for the difference in secretion between these two patients is that the homozygous alteration of a cysteine residue in patient 5 (*p.Cys267Tyr*) has a more dramatic effect on protein folding and trafficking by altered disulfide bridge formation compared with the glutamic acid to lysine alteration in patient 1. However, two previously reported *FBLN4* mutations, *p.Glu57Lys*[13] and *p.Arg279Cys*, in combination with *c.1070_1073dupCCGC*,[12] show a complete absence of the mutant

protein in the extracellular matrix; the latter was confirmed in this report by immunohistochemical staining of fibulin-4 on aortic tissue. This indicates that the diminished amount of mutant fibulin-4 present in the extracellular matrix of patient 1 is the first example of a patient with *FBLN4* mutation with the production and secretion of the mutant protein. This might be in accordance with the longer survival of the patient. Given these observations, we suggest that mutations in *FBLN4* impair the stability and/or secretion of fibulin-4, similar to previous findings for fibulin-5 mutations.[34] The resulting decrease of protein in the extracellular matrix leads to altered interactions with fibulin-4 binding partners and, subsequently, to impaired elastogenesis. Fibulin-4 is known to associate with tropoelastin, possibly connecting elastin to microfibrils to form elastic fibers.[30] Second, fibulin-4 also binds strongly to the N-terminal region of fibrillin-1 in the presence of Ca^{2+}.[35,36] As suggested by Ono *et al*,[36] fibulin-4 competes with latent TGFβ binding proteins (LTBPs) for binding to fibrillin-1. As such, this fibulin protein may have a role in the modulation of LTBP sequestration by the extracellular matrix, an event important in the regulation of the bioavailability of TGFβ for activation and signaling. As a result of impaired mutant fibulin-4 secretion and/or stability and the resulting impaired elastogenesis, we believe that LTBP is no longer able to bind the fragmented elastic fibers. The TGFβ–latency-associated peptide complex is released and TGFβ is subsequently more prone to activation. An analogous mechanism has previously been suggested to occur in MFS and LDS.[10,31,37] In addition, the resulting decrease of mutant fibulin-4 protein in the extracellular matrix may lead to defective smooth muscle terminal differentiation, as recently suggested by Huang *et al*.[11] They hypothesized that fibulin-4 deficiency not only results in aneurysm formation through defective elastic fiber formation but also through extracellular regulation of smooth muscle cell differentiation genes.

To investigate whether increased TGFβ signaling is involved in the pathogenesis of autosomal recessive cutis laxa type I in humans, as predicted on the basis of observations of impaired TGFβ signaling in *Fbln4-/-* mice by Hanada *et al*,[6] we assessed the level of pSmad2 and CTGF expression in the aorta and lung and subsequently performed pSmad2 immunoblotting on fibroblast cultures before and after TGFβ stimulation. TGFβ-stimulated fibroblast cultures of patients with *FBLN4* mutations in this study showed an increased and more intense level of phosphorylated Smad2 compared with controls. This finding is in line with an '*in vivo*' increase in TGFβ signaling demonstrated in the aorta and lung of fibulin-4-deficient patient 4. The increased TGFβ signaling in aortic tissue of *FBLN4* mutation-positive patients confirms the key role of this signaling pathway in the pathogenesis of aortic and arterial aneurysms and tortuosity, as demonstrated previously in other human diseases such as MFS, LDS and ATS.[8–10,32] As already shown in Marfan mouse models, this finding offers the potential for treatment by blocking TGFβ signaling through the use of angiotensin receptor type 1 blockers such as losartan.[38]

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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