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

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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 elastin 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-like domains. An RGD motif, a specific cell attachment sequence interacting with αvβ3 and αvβ5 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.
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 \textit{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 fundus 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 (\textit{ELN}) was excluded as the site of primary mutation using fluorescence \textit{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 \textit{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 \textit{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
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 \(\beta\) 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 fibrulins, such as fibrulin-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 \textit{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 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.
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 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 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'-TTGGTTGCTTTCCATCCATCTG-3'; chFBLN5-F2, 5'-CACCGATCTCCAGGCCTC-3'; chFBLN5-R2, 5'-TGAAACGCAATCTTCTCCAAG-3'; chFBLN5-F3, 5'-ACGGCTCTTTCATCTGCCG-3'; chFBLN5-R3, 5'-GCCTTTACCATCTTGGTACC-3'; chFBLN5-F4, 5'-ACCTCTTACCATCTTGGTACC-3'; chFBLN5-R4, 5'-AAATGAGAGCGCAGCTCGGG-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'-AGATGTGAAAGCTGTGAGG-3'; ghFBLN5-R1,
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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