Aortic Aneurysmal Disease and Cutis Laxa Caused by Defects in the Elastin Gene

Running title: ELN Mutations in Aortic Aneurysms

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Abstract

Introduction: Cutis laxa (CL) is an acquired or inherited condition characterized by redundant, pendulous and inelastic skin. Autosomal dominant CL has been described as a benign disease with minor systemic involvement.

Methods and Results: We studied a family with autosomal dominant CL and a young female with sporadic CL, both with variable expression of an aortic aneurysmal phenotype ranging from mild dilatation to severe aneurysm or aortic rupture. Histological evaluation of aortic aneurysmal specimens indicated classical hallmarks of medial degeneration, paucity of elastic fibers, and an absence of inflammatory or atherosclerotic lesions. Electron microscopy showed extracellular elastin deposits lacking microfibrillar elements. Direct sequencing of genomic amplimers detected frame shift mutations in exon 30 of the elastin gene in affected individuals, but did not in 121 normal controls. The expression of mutant elastin mRNA forms was demonstrated by reverse transcriptase-polymerase chain reaction analysis of transcripts from CL fibroblasts. These mRNAs coded for multiple mutant tropoelastins, including C-terminally truncated and extended forms, as well as for molecules lacking the constitutive exon 30.

Conclusion: We conclude that ELN mutations may cause severe aortic disease in patients with CL. Thus, regular cardiac monitoring in CL is necessary to avert potential fatal aortic rupture.
Introduction

Early clinical examination and review of published cases of CL led to the conclusion that recessive CL was associated with high morbidity and mortality of cardiopulmonary causes including pulmonary emphysema and aortic aneurysms, whereas autosomal dominant CL was free of grave systemic lesions and was associated with a normal lifespan. Heterozygous mutations in the elastin gene (ELN) have been shown to cause autosomal dominant CL. The phenotypic spectrum of this condition remains poorly defined as only 5 patients have been described with detailed clinical information and positive mutational results.

We have recently described a family with autosomal dominant CL caused by an unusual, partial tandem duplication in ELN. Associated systemic conditions included inguinal hernias and severe emphysema that led to end stage respiratory failure requiring a lung transplant in one of the affected family members. This showed for the first time that ELN mutations could lead to fatal pulmonary complications. In the present study, we report a family with autosomal dominant cutis laxa and a sporadic CL patient with ELN mutations and variable expression of a thoracic aortic aneurysmal phenotype ranging from mild dilatation to severe aneurysm requiring aortic root replacement or leading to aortic rupture early in adulthood.

Methods

Human subjects

Blood samples, skin biopsies and surgically removed tissue specimens were obtained from study participants after informed consent. Normal control tissue samples were provided by the Cooperative Human Tissue Network which is funded by the National Cancer Institute. Other investigators may have received specimens from the same subjects. This study was approved by the Committee on Human Studies (IRB) of the University of Hawaii and of Washington University in St. Louis.

Mutational analysis

After isolation of nuclei from whole blood, DNA was purified by proteinase K digestion and phenol extraction. Each exon of ELN with flanking intronic sequences was amplified using polymerase chain reaction (PCR) as described earlier. Amplimers were directly sequenced on both strands using the BigDye terminator cycle sequencing chemistry (Applied Biosystems, Foster City, CA). A panel of 121 normal control individuals was genotyped for mutations 2114_2138del and 2159delC using denaturing high performance liquid chromatography (dHPLC, Wave, Transgenomic, Omaha, NE).

Expression studies

For the analysis of allelic expression, 3 µg of total RNA was used to generate first-strand cDNA using a Superscript preamplification system (Invitrogen, Carlsbad, CA) with oligo-dT primers. First strand cDNA was amplified using oligonucleotide primers 5’-AGCCAAAGCTGCTGCCAAAG-3’ and 5’-TTCTTTCCGGCCACAACCT-3’ complementary to exons 29 and the 3’-UTR, respectively. The exon 29 primer was radiolabeled using γ-P[33]-ATP to generate radioactive RT-PCR products, which were separated by denaturing polyacrylamide gel electrophoresis. Products were quantitatively analyzed using a Typhoon 9410 phosphorimager (Amersham, Piscataway, NJ). To establish the dynamic range of our RT-PCR assay, we conducted experiments at various...
cycle numbers. The amplification was found to be exponential up to 35 cycles. Subsequent reactions were conducted at 22-23 cycles to ensure quantitative recovery of RT-PCR products representing various isoforms. Products that achieved, in at least one sample, >1% of the total signal were excised from the gel, re-amplified, and analyzed by direct DNA sequencing to uncover the primary structure of each elastin mRNA isoform.

**Results**

**Clinical description**

We studied a three-generation family of Japanese and German ancestry (CL-16, Figure 1). At an age of 38 y, II.2 had a large aneurysm of the sinuses of Valsalva and of the ascending aorta as demonstrated by magnetic resonance imaging and CT angiography (Table 1), which was repaired using an aortic graft and valvuloplasty. One year later, he developed aortic insufficiency requiring aortic valve replacement. His skin was of normal texture with barely noticeable skin laxity, which had been reported to be more evident on the lower face and submandibular area during childhood. He had a single palmar crease bilaterally and limited ability to supinate both forearms. II.3 died at age 26 y of aortic dissection with no other apparent systemic involvement. He had no visible skin laxity in photographs taken during childhood and adulthood. Both I.1 and III.1 had mildly dilated aortic root (Table 1), mild aortic insufficiency, and obvious CL, with a velvety texture to the skin by palpation and with redundancy of skin on the face, submandibular area, trunk and to a milder degree on the extremities. III.1 is treated with atenolol 50 mg daily. Pulmonary function testing of III.1 at the age of 11 yrs indicated overall normal function (forced expiratory volume in 1 sec: 96% predicted) with mildly elevated residual volume (152% predicted). He also had a history of prolonged wound healing. I.1, II.2 and III.1 all had history of inguinal hernias repaired surgically.
Table 1. Aortic diameters in mutation carriers in CL-16

<table>
<thead>
<tr>
<th></th>
<th>Ann (mm)</th>
<th>Sinus (mm)</th>
<th>Asc (mm)</th>
<th>Trans (mm)</th>
<th>Desc (mm)</th>
<th>Aortic Insuff</th>
<th>Age (yr)</th>
<th>Height (cm)</th>
<th>Wt (kg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>III.1</td>
<td>26</td>
<td>34</td>
<td>26</td>
<td>11</td>
<td>10</td>
<td>mild</td>
<td>8</td>
<td>138.5</td>
<td>56.7</td>
</tr>
<tr>
<td>II.2</td>
<td>*66-72</td>
<td>*52-58</td>
<td></td>
<td></td>
<td></td>
<td>severe</td>
<td>39</td>
<td>177.8</td>
<td>99.9</td>
</tr>
<tr>
<td>I.1</td>
<td>23</td>
<td>30</td>
<td>34</td>
<td>26</td>
<td>20</td>
<td>mild</td>
<td>71</td>
<td>141.5</td>
<td>63.5</td>
</tr>
</tbody>
</table>

Notes. Ann = aortic annulus, Asc = ascending aorta, Trans = transverse aorta, Desc = descending aorta, Insuff = insufficiency, Wt = weight. Normal values for aortic sinus measurements should not exceed 32 mm up to 2 m^2 body surface area (II. 2) and at 1.6 m^2 (III.1 and I.1) the normal range is 22-29 mm. Supra-aortic ridge or ascending aorta long axis view echocardiographic measurements should not exceed 25 mm and 28 mm respectively at up to 2 m^2. II.2 aortic sinuses measured 66-72 mm on transesophageal echo and ascending aorta 52-58 mm on CT depending on view. Normal aortic root for age 46 +/- 13 years = 32 +/- 3mm.

An unrelated Singaporean girl of Chinese descent with no family history of CL (CL-13, Figure 1) was noticed at birth to have marked laxity of the skin. Physical features included loose folds of redundant skin over the face and jowls, trunk, and over the upper and lower limbs. The hair was sparse, and she had tear-shaped eyes, with prominent anteverted ears, a long philtrum, and a flattened nasal bridge. At the age of 5 years 9 months an echocardiogram demonstrated significant dilatation of the aortic root at the level of the sinuses of Valsalva with a measured diameter of 2.47 cm (normal < 2.2 cm for her body surface area). There was no aortic regurgitation and the ascending aorta was of normal size. She was treated with atenolol 25 mg daily to delay progression of aortic dilatation and development of aortic aneurysms.

Pathology

Aneurysmal aortic samples (aAo) obtained from family CL-16, patient II.2 showed reduced wall thickness (1-1.5 mm), approximately 50% of adjacent non-aneurysmal (nAo) tissue (2.5-3 mm). Hematoxylin-eosin staining of aAo showed focal paucity and clumping of smooth muscle cells (Figure 2C) characteristic of medial degeneration, and nAo patient samples indicated slightly reduced cellularity (Figure 2B) compared to a normal control (Figure 2A). Inflammatory infiltrates or atheromatous changes were not apparent in any of these specimens. Elastic staining in normal aorta showed dense, wavy elastic lamellae (Figure 2D). In contrast, elastic fibers appeared stretched and fewer in nAo (Figure 2E) and were destroyed leaving only a few scattered fragments in aAo (Figure 2F). Hart’s staining of skin samples from CL-13, II.1 (Figure 2H) showed thin elastic fibers, whereas staining intensity in CL-16, III.1 (Figure 2I) was comparable to control (Figure 2G). In both CL skin samples the normal waviness of deep elastic fibers was diminished. Electron microscopy in controls demonstrated abundant microfibrillar bed surrounding dermal elastic fibers (Figure 2J). In contrast, elastic fibers in the skin of CL-16, III.1 lacked associated microfibrils (Figure 2K).

Mutational results

Direct sequence analysis of ELN in CL-16, III.1 uncovered a 25 bp deletion in exon 30, 2114_2138del. This defect was present in all affected members of the family available for testing, including I.1 and II.2. The mutation was not found in II.1. Direct sequence analysis also uncovered mutation 2159delC, also in exon30 of ELN, in CL-13, II.1 which was absent in both of her unaffected parents (I.1 and I.2) indicating that 2159delC was a
de novo mutation. Screening of the 121 normal control individuals was negative for both 2114_2138del and 2159delC.

Expression of mutations 2114_2138del and 2159delC

To analyze the effect of mutations 2114_2138del and 2159delC on elastin biosynthesis, we performed RT-PCR experiments using total RNA from mutant and normal control fibroblasts (Figure 3A). Normal cells expressed two mRNA species as a result of alternative splicing of exon 32 (Figure 3B). The same products were also observed in CL cells corresponding to products of the normal allele in heterozygotes. CL cells also expressed multiple mutant-specific mRNA forms that had similar structures in both CL-13 and CL-16 (Figure 3C-E). Inclusion of either of the two mutations in the full length mRNA resulted in mRNA with a frame shift and premature termination codons in a sequence encoded by exon 32 (Figure 3C). Inclusion of the mutations and the removal of exon 32 by alternative splicing resulted in the same frame shift with a C-terminally extended open reading frame (Figure 3D). Finally, both mutations induced illegitimate skipping of the constitutive exon 30 (Figure 3E). The relative proportion of these mutant mRNAs were different between CL-13 and CL-16. The C-terminally extended product was the most abundant in CL-13 (Figure 3D) and exon 30 skipping products were the most abundant in CL-16 (Figure 3E).

Discussion

Our studies demonstrate for the first time that ELN mutations associated with cutis laxa may cause aortic lesions ranging from mild dilatation to severe aneurysm and rupture of the aortic root. Variable expression of CL, hernias, and aortic lesions was observed in a family with autosomal dominant CL, and mild aortic dilatation was found in a sporadic patient with severe CL. The aortic pathology associated with this condition is medial degeneration, characterized by dramatic loss of elastic lamellae and smooth muscle cells and a lack of atherosclerotic and inflammatory lesions. These findings emphasize the importance of cardiovascular monitoring to prevent possible fatal rupture of the aortic root in CL patients.

The aortic lesions documented in this study are highly similar to the aortic disease associated with Marfan syndrome (MFS). Both are characterized by (1) variable expression, (2) localization of the lesions to the aortic root, and (3) the pathology of medial degeneration. Aortic root aneurysms in MFS are thought to be caused by the loss or weakening of microfibrils anchoring medial smooth muscle cells to elastic lamellae, leading to phenotypic alteration of smooth muscle cells, destruction of lamellae, and a loss of aortic wall integrity. Electron microscopic observation of disrupted microfibril-elastin interactions in our study suggest that aortic lesions in CL may have a shared pathomechanism with aneurysms in MFS in addition to the clinical and pathological similarities demonstrated here.

How can mutations in ELN disrupt microfibril-elastin interactions? Our recent studies demonstrating synthesis and matrix incorporation of mutant tropoelastin in CL patients have provided an insight into this question. Deposition of a mixture of normal and mutant elastin may increase the protease susceptibility of this protein polymer leading to the destruction of molecules important for microfibril-elastin interaction. Alternatively, mutant elastin may interfere with such interactions in a dominant negative manner. The present study is consistent with the synthesis of an abnormal protein, as mRNA products of the mutant and normal alleles were found in equal abundance. Both mutations reported here result in the synthesis of several stable mutant mRNA species, including isoforms...
that encode an extended hydrophilic C-terminal missense peptide sequence, lack constitutive exon 30, or have truncated open reading frames.

Our studies provide evidence for the involvement of ELN mutations in the development of CL and severe thoracic aortic aneurysms. Dramatic disappearance of elastic fibers in aneurysmal lesions also implicates ELN as a candidate gene for inherited aneurysmal diseases. It remains to be shown whether ELN may be mutated in patients with familial, nonsyndromic thoracic aortic aneurysms or whether ELN may be a susceptibility gene for aneurysmal diseases of complex inheritance. The latter is supported by some genetic mapping and association studies on intracranial aneurysms.

Acknowledgements

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References

Figure Legends

Figure 1. Variable expression of CL and associated lesions in families CL-16 and CL-13

Figure 2. Aortic and dermal pathology in CL

Hematoxylin-eosin staining of a normal control (A, 29 y Caucasian male), non-aneurysmal (B, nAo), and aneurysmal (C, aAo) segments of the aorta of patient CL16, II.2 (38 y). Focal cell paucity (arrows) and cell clumping (arrowheads) are seen in aAo (C). Verhoeff van Giesson (VVG) elastin stain of sections a from normal control (D, 15 y African American male) shows dense, wavy elastic lamellae. In nAo, the elastic fibers are irregular and stretched (E, arrows). In aAo only fragments of the lamellae remain (F, arrows). Hart’s elastin stain (G-H) of a normal skin section (G, 9 y male) shows thick, horizontal, undulating elastic fibers in the deep dermis (arrows) and fine, perpendicular fibers in the papillary dermis. In the skin of CL-13, II.1 (H, 6 y Asian female) the deep dermal elastic fibers are thin and straight (arrows), whereas in CL-16, III.1 (I, 12 y male) the fibers are of normal thickness but lack the normal wavy character (arrows). Electron microscopy of the skin in normal control skin (J) shows abundant microfibrillar component, both at the periphery (Mf) of, and as inclusions in, a mature elastic fiber (El). In contrast, elastic fibers (El) in the proband’s skin (CL16, III.1) are not associated with the appropriate amount and localization of microfibrils (K). A small bundle of microfibrils is seen disjoined from the elastic fiber (Arrowhead). Magnification bars (A-I): 100 µm (J,K): 483 nm.

Figure 3. Mutant elastin mRNA isoforms in CL-13 and CL-16

A, Reverse transcriptase-polymerase chain reaction (RT-PCR) amplification of RNA samples from normal control and mutant fibroblasts. Lane 1: plasmid positive control. Lane 2: no template negative control. Lane 3: normal control sample. Lane 4: no reverse transcription negative control. Lane 5: mutant (CL16, II.2) sample. Lane 6: mutant (CL13, II.1) sample. Positions and sizes of fragments are shown on the right in base pairs. RT-PCR products with an abundance of >1% of total are numbered on the left. B, Domain structure of normal tropoelastin. C, Domain structure of predicted mutant tropoelastins containing a premature termination codon in exon 32 (products 2 and 3). Exons (30-32) encoding a C-terminal missense peptide as a result of the frame shift introduced by the mutations are shown in red. The sequence of the missense peptide sequence is shown below the diagram for each mutation. D, Domain structure of predicted mutant tropoelastin with an extended open reading frame. Sequences (exons 30, 31, 33, 34 and 3’-UTR) encoding a C-terminal missense peptide as a result of the frame shift introduced by mutations 2159delC (product 5) and 2114_2138del (product 7) are shown in red. E, Domain structure of predicted mutant tropoelastin isoforms lacking constitutive exon 30 (products 6 and 8). Percent values indicate the abundance of each isoform relative to total elastin mRNA abundance in the corresponding samples (100%).
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For The Blind to support gene mapping of autosomal dominant nystagmus.

1 Hemmes GC. *Over hereditaire nystagmus.* Wageningen: H Veerman & Zonen, 1924.

Corrections

In the paper by Richards *et al* on 'Detailed genetic mapping of the von Hippel-Lindau disease tumour suppressor gene' (*J Med Genet* 1993;30:104-7), an important collaborator, Dr Per Enblad, was inadvertently omitted from the authorship. The correct authorship is as follows.


Cambridge University Department of Pathology, Cambridge, UK; *Laboratory of Immunobiology, National Cancer Institute, Frederick Cancer Research Facility, Frederick, USA; †Erasmus University, Rotterdam, The Netherlands; §University of Uppsala, Sweden; Division of Community Medicine, Memorial University of Newfoundland, Canada; ||Yorkshire Regional Genetics Service and ICRF Genetic Epidemiology Laboratory, Leeds, UK; §Surgery Branch, National Cancer Institute, USA.

In the paper by Padayachee *et al* on 'Mapping of the X linked form of hyper IgM syndrome (HIGM1)' (*J Med Genet* 1992;30:202-5), the primer sequence for DXS10221 under the heading of oligonucleotide primers was referenced Luty *et al*. This is incorrect and should be: