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Deletion of the Paired $\alpha 5(\text{IV})$ and $\alpha 6(\text{IV})$ Collagen Genes in Inherited Smooth Muscle Tumors

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The gene encoding $\alpha 6(\text{IV})$ collagen, *COL4A6*, was identified on the human X chromosome in a head-to-head arrangement and within 452 base pairs of the $\alpha 5(\text{IV})$ collagen gene, *COL4A5*. In earlier studies, intragenic deletions of *COL4A5* were detected in a subset of patients with Alport syndrome (AS), a hereditary defect of basement membranes. In some families, AS cosegregates with diffuse leiomyomatosis (DL), a benign smooth muscle tumor diathesis. Here it is shown that patients with AS-DL harbor deletions that disrupt both *COL4A5* and *COL4A6*. Thus, type IV collagen may regulate smooth muscle differentiation and morphogenesis.

Basement membranes (BMs) compartmentalize tissues and provide important signals for the differentiation of the cells they support. Type IV collagen, the major structural component of BM, is a triple-helical molecule composed of three α chains (1). To date, five genetically distinct type IV isoforms have been described in mammals

(1–4). The $\alpha 1(\text{IV})$ and $\alpha 2(\text{IV})$ chains encoded by *COL4A1* and *COL4A2*, respectively, are ubiquitous, whereas $\alpha 3(\text{IV})$, $\alpha 4(\text{IV})$, and $\alpha 5(\text{IV})$ have restricted tissue distributions (3, 5). On the basis of sequence similarities, the chains fall into two classes: $\alpha 1(\text{IV})$, $\alpha 3(\text{IV})$, and $\alpha 5(\text{IV})$ compose the $\alpha 1$ -like class, and $\alpha 2(\text{IV})$ and $\alpha 4(\text{IV})$ compose the $\alpha 2$ -like class. The human genes for the $\alpha 1(\text{IV})$ and $\alpha 2(\text{IV})$ chains are located in a head-to-head configuration on chromosome 13 (6), and the $\alpha 3(\text{IV})$ and $\alpha 4(\text{IV})$ genes are similarly arranged on chromosome 2 (7). Thus, it appears that the type IV collagens evolved by duplication of an ancestral α chain gene, giving rise to a pair of α chain genes with closely apposed 5' ends. The pair presumably underwent additional duplication, giving rise to the ancestors of the $\alpha 1(\text{IV})$ -

$\alpha 2(\text{IV})$ and $\alpha 3(\text{IV})$ - $\alpha 4(\text{IV})$ gene pairs. We predicted that the $\alpha 5(\text{IV})$ gene, a member of the $\alpha 1(\text{IV})$ -like class, might also be paired with an $\alpha 2(\text{IV})$ -like gene that had not yet been identified.

Mutations in *COL4A5* are estimated to be present in ~50% of X-linked AS cases (8). In males AS is characterized by progressive renal failure, sensorineural deafness, and ocular lesions; female carriers are mildly affected. In some families AS cosegregates with DL (9), a benign proliferation of smooth muscle in the esophagus, female genitalia, and trachea. Both sporadic and hereditary cases have been reported (10). We have shown that AS-DL patients have deletions that include the 5' end of *COL4A5* (11), whereas AS patients without DL have internal deletions or point mutations of *COL4A5* (8). These results suggested that DL is caused by the deletion of an unidentified gene located upstream of *COL4A5*.

To isolate the putative type IV collagen gene upstream of *COL4A5*, we screened an X-chromosome library with JZ-4, an $\alpha 5(\text{IV})$ cDNA clone (4), and isolated a 14.1-kb clone, λ LA226 (Fig. 1). It contained exon 1 of *COL4A5* and an upstream 2.8-kb Hind III fragment, LA226-H6, that displayed cross-species hybridization. We therefore used LA226-H6 to probe an adult kidney cDNA library. Three identical clones, JZK-1, JZK-2, and JZ-3, contained an open reading frame (1643 bp) encoding a 21-amino acid signal peptide, a 25-amino acid noncollagenous segment, and a 502-amino acid collagenous domain with nine interruptions (Fig. 2) that are believed to confer flexibility in type IV collagens. The deduced translation product, which we have termed $\alpha 6(\text{IV})$, is a type IV collagen that has not previously been detected genetically or biochemically (12). Sequence analysis clearly places $\alpha 6(\text{IV})$ in the $\alpha 2(\text{IV})$ -like class (Fig. 2). The head-to-head arrangement of *COL4A5* and *COL4A6* resembles that of *COL4A1* and *COL4A2*.

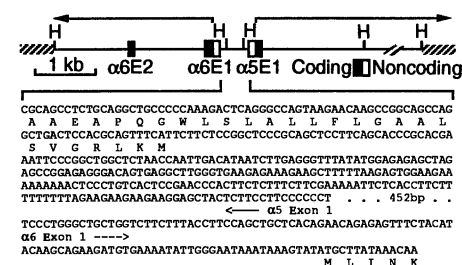


Fig. 1. Restriction map of λ LA226 which contains the 5' ends of both *COL4A6* and *COL4A5*. The striped bars represent phase arms; H, Hind III. The DNA sequence of the region containing the first exon of each gene is shown as well as the deduced amino acid sequences of the first exons (24).

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α6 1 MLIN
α2 1 MGRDQRAVAGPA-RR
α1 1 MGRPLRSV
α5 1 MKLR GVS-AA

5 K L K L L L V L T L C L T E E L A A A G K S Y K P G C G Q C S G S Q C F F
16 W-L-GT--VGF LAQSLV--V-KFDV-----R-----G--Y
8 W-L--PAA-L-H--HSR-AA--GG-A-SG--K-D-HG
11 G-F-- A-S-WQGP-E-AA--G--Y CSPSGK-D-SG

45 E K G A R G R P G P I G I Q G P T G Q G T G S T G L S G L K G E R G F P G L
56 ---G-Q---V-P-YN--P-LQ-PP-Q-P--DK-ER-A
44 V--K-ER-LP-L-VI-PP-MQ-FP-PQ-PR-QK-DT-E
43 I--EK-ER-FP-LE-HP-LP--P-PE-PP-PR-QK-DD-I

85 L G P Y G K G D K G P M G V P G F L G I N G I P H G P G Q P R G P P G L D
96 P-VT-----V-AR--S--P-AD-----G-----R--Y
84 P-LP-T--TR--P-AS-YP-NP-L-I---D--P-----IP
83 P--P-----IR--P-DL--P-TP-L--M--HD-AP--Q-LP

125 G C N G T Q G A V G P P G D P G Y L L G P P G L P Q K G S K G D V P L A P
136 -----DS-PQ--P-SE-FT-----PQ--P--E-YAL-
124 -----K-ER-PL--P-L-FA-N--P--L-P-M-----G E I L
123 -----K-ER-----S-P-F-----P--IP-M-E-G S I L

165 **G S F K** G I K G D P L G P L D G I T G P Q G A G F P G A V G P A G P P G
176 **K E E R D Y R** -E--F--F-Q-P--R-L-HV-QM--V-A--A
164 **H V P G M L L** -E-R-F--IP-TP-P--P-L--Q--P--P-FT--
163 **M-S-L-P** -N-N-Y--PP--Q-LP-PT-I--PI--P-----

I

203 L Q G P P G P P G L G P D G N M G L G E Q G E K V K G D V L G P G P A G P
215 RP-----K-QQ--R--Y-V-E-----Q--N-I
204 PP-----P-EK-Q--S--P-D-----Q-VS-P-V
202 M-----LP-K--N--P--E-E-EQ-Q--P--P

II

243 P S T G E L F M G F P K K K G S K G E P G K G F P G I S G P P G P F
246 S D - L H P I A P G T F T F H P D O Y K -E--SE-EP-IR-IS
244 G O A O V I K A D G -A T K -E--EK-EP-FQ-M
242 Q O I S - O K R E I D V E F O -DQ-L--DR-P--PP-IR-P-GPP

III

280 G L I G T G E K G E K G E K I P L G P R G P M G S E G V Q G P P G Q Q K
282 L K -E-E-IM-FP-LL-R-Y--S-EK-SP-QK-SR-LD-Y-P
273 -V-EK--P-KP-PR-K--KD-CK-EP--P-PP-E--YP-L
282 -EK-----P--P-KR-K--KD-EN-QP-IP-L--DP-Y

IV

302 K G T L G P P L G N G F Q I E G Q K G I D L G P P V E I D G A V I S G
332 D-PR-PK-EA-DP-PP-LP **A Y S P S L A K**
313 I-RQ-QP-EK-EA-PP-PP-VI **I G T G P L E K G**
321 P-E-P-RD-EK-QK-DT-PP-PP-- **V I R P P T G T I T E K G**

V

360 N P G D P G V P L G L K G D E G I Q L R G P S G V P L P A L S V P G A
362 A R ---F--AQ-EP-SQ-EP-DP-LP-P---S I G D -D O R R
345 E R -Y-T--PR-EP-PK-FP--P-QP-P--V E -Q A--
360 -I-L--L--EK-ER-FP-----PP-LP-P--A A V M -P--P

VI

400 L G P Q G F P L K D G N C P G R T T I G A A G L P G R D L G P P G P G
402 -LP-EM-P--FI-D-IP A I A Y G -P-L-L--P--P--L
384 P-FP-ER-E--R-F--T S L P -P S -R D -L P -P--S--
399 P-FP-ER-Q---E-P--I S I R -PP--D-QP-A--L--G

VII

438 P G P P S P E F E T T L H N K S E G P P L R G E Q P G K N L G L K I G K
441 ---G-DG F L E G L K G A K -R A -F P -L P -S P -A R -P K -W--
422 -Q-G Y T T I N G V E C O R G P -D Q -P P -I P -Q P -F I -E -E
437 ---A G -H I P P S D E I C P P -P--PP-S-P--D--L Q -E Q -V--

VIII

478 D S G F C A C D G G V P P T G P P E G P G P P W G L I G L P G L K G A R G
480 -A-E-R-TE-DE A I K -L-L-L--K-A-I-IP-E--R--DK-
462 QK-ESCLICLDIDYGR-----PQ--E-I-FN-Q-A--D--
476 -K-DT-FCN I C T G I S -G--L-L--LP-PP-SL-FP-QK-

IX

513 D R G S G A O G P A G A P I G V L G P S G P K G K K G 548
520 P-QH-LP-PK-LK-VP-NI-AP-----A-D 551
502 LP-RD-VA-V-P-QP-LI-QP-A--EP-E 533
516 EK-OA-T-T-K-L-TP-AP-AP-PP-S-E 547

Fig. 2. Deduced amino acid sequences of the cDNA clone JZ-3 of the human $\alpha 6(\text{IV})$ chain and comparison of JZ-3 with the amino acid sequences of the human $\alpha 1(\text{IV})$, $\alpha 2(\text{IV})$, and $\alpha 5(\text{IV})$ chains (4, 23). Dashes indicate amino acid identity, and conserved cysteine residues are indicated by arrows. The locations of cysteines in the mature chains are identical in $\alpha 6(\text{IV})$ and $\alpha 2(\text{IV})$. Interruptions in the collagenous repeat are underlined and numbered (I through IX). The GenBank accession number is L22763.

ity, we performed Southern (DNA) blot analysis (Fig. 4) on 140 AS patients, including four unrelated AS-DL patients (13), with JZ-3. We saw an abnormal pattern only in the AS-DL patients. There was a loss of bands in males and a 50% reduction in the intensity of some bands in females, but the pattern was complex. We therefore used a fragment, JZ-3-FR5, containing exons 1 to 4 of COL4A6 to map the deletions more precisely (Fig. 4). The 1.4-kb and 5.6-kb Eco RI fragments, which contain exons 1 and 2 of COL4A6, were absent in males and reduced in intensity by ~50% in females; exons 3 and 4 were intact. Hybridization with JZ-4 demonstrated the loss of exon 1 of COL4A5. Exons 2 to 10 of COL4A5 were present in four of the five patients. Therefore, the smallest AS-DL deletions involve part of intron 1 and all of exon 1 of COL4A5, the intergenic region, and exons 1 and 2 and part of intron 2 of COL4A6.

We studied the distribution of the $\alpha 5(\text{IV})$ and $\alpha 6(\text{IV})$ transcripts in the fetus (Fig. 3B). An $\alpha 6(\text{IV})$ mRNA of ~ 7.0 kb was found in meninges and esophagus and was just detectable in fetal choroid plexus and stomach. An ~ 7.0 -kb transcript of $\alpha 5(\text{IV})$ was present in many tissues but was most abundant in choroid plexus, meninges, and esophagus.

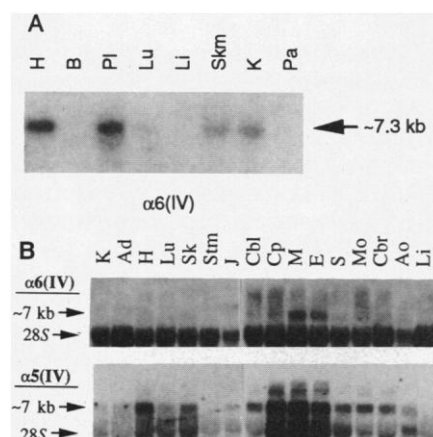


Fig. 3. (A) Northern (RNA) blot analysis of tissues from a normal adult. Probe JZ-3, representing $\alpha 6$ (IV) sequences, was hybridized to a filter containing $\sim 2 \mu\text{g}$ of polyadenylate-enriched RNA (per lane) that had been separated by agarose electrophoresis. **(B)** Northern blot analysis of tissues from a 24-week human fetus. Probes JZ-3 and PL-31 were serially hybridized to a filter containing $\sim 20 \mu\text{g}$ of total RNA in each lane except for samples from aorta ($\sim 9 \mu\text{g}$) and jejunum ($\sim 10 \mu\text{g}$). We separated RNAs as in (A). Ad, adrenal; Ao, aorta; B, whole brain; Cbr, cerebrum; Cbl, cerebellum; Cp, choroid plexus; E, esophagus; H, heart; J, jejunum; K, kidney; Li, liver; Lu, lung; M, meninges; Mo, medulla oblongata; Pa, pancreas; Pl, term placenta; S, stomach; Skm, skeletal muscle; and Stm, striated muscle.

We found deletions of both COL4A5 and COL4A6 in all four independent AS-DL kindreds. How do these null mutations cause DL? Interactions between cells and the underlying BM substrate play a vital role in embryonic morphogenesis (14). Signals from BM proteins, transduced by members of the integrin family of cell surface receptors, regulate cell growth and differentiation and influence cell shape by affecting the cytoskeleton. Several BM components interact with integrins (15) and can affect differentiation of epithelial, endothelial, and mesenchymal cells. Type IV collagens contain binding sites within the triple-helical (16) and NC1 domains (17) for several cell types including myocytes (18). In the presence of antibodies to a β integrin, chicken embryo myoblasts continue to replicate, fail to fuse, and have abnormal morphology (19). Although the cited studies are based on collagen sources rich in the ubiquitous $\alpha 1(\text{IV})$ and $\alpha 2(\text{IV})$ chains (20), the data presented here suggest that the $\alpha 5(\text{IV})$ and $\alpha 6(\text{IV})$ chains may play similar roles in cell-matrix interactions in tissues involved in the AS-DL syndrome; their absence may disrupt normal morphogenesis and lead to uncontrolled proliferation of distorted smooth muscle cells.

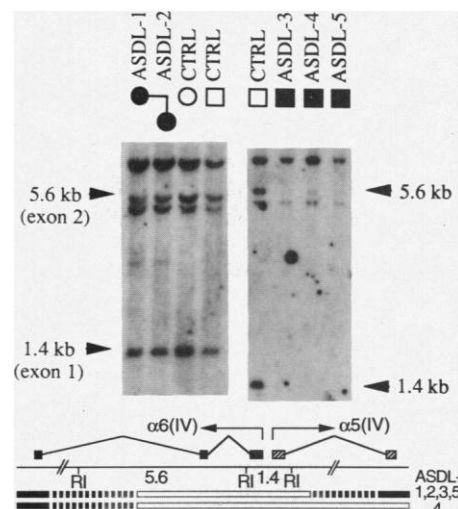


Fig. 4. Analysis of collagen gene deletions in five patients with AS-DL. Genomic DNA samples (~5 µg) from patients (filled symbols) and controls (open symbols) were digested with Eco RI and hybridized with JZ-3-FR5, a cDNA fragment containing the first four exons of $\alpha 6(\text{IV})$. Squares indicate males and circles indicate females. In the panel at the bottom, the three exons of $\alpha 6(\text{IV})$ and two exons of $\alpha 5(\text{IV})$, shown as black and striped boxes, respectively, are placed on a genomic map of Eco RI sites (RI). The open bars below show the minimum extent of the deletion in each AS-DL patient. The filled bars show the minimum extent of the nondeleted regions. The shaded bars represent ambiguities in mapping the deletion boundaries.

Mutations of neither COL4A5 alone nor COL4A6 alone have been observed in AS-DL. It is possible that only the $\alpha 6(\text{IV})$ chain is critical for normal smooth muscle differentiation. If so, our failure to observe mutations of COL4A6 alone in AS-DL might merely reflect the small sample size. Linkage studies (21) have shown that X-linked AS mutations are all tightly linked to markers in the Xq22 region where both COL4A5 and COL4A6 are located. COL4A6 is the probable site for the ~50% of X-linked AS mutations that have not been found in COL4A5. Therefore, the absence of DL in these patients suggests that simultaneous mutation of both COL4A5 and COL4A6 is required for the development of DL.

Attention has previously been focused on the role of type IV collagen in adhesion and motility of cells during tumor invasion (22). Here we show that constitutional mutations in type IV collagen can result in cell proliferation in benign tumors. Mutations in other BM components, whether constitutional or acquired, may also participate in the pathogenesis of other benign tumors.

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- The patients ASDL-1 and ASDL-2 are related females. ASDL-1 had persistent hematuria and esophageal and genital leiomyomas. Her brother had AS with cataracts and hearing loss and died

- at age 23 after an unsuccessful renal transplantation. He suffered from an undiagnosed swallowing difficulty and "narrow esophagus." Her mother also had unexplained swallowing difficulty. One sister died at age 37 from myosarcoma of the brain. Two other sisters are healthy. Her daughter, ASDL-2, developed hematuria at age 9. Ultrastructural changes in glomerular basement membrane are typical of AS. She has bilateral cataracts and DL. Patients ASDL-3, -4, and -5 have been described previously (11). ASDL-3 is a male with biopsy-proven AS, deafness, cataracts from age 2, and symptomatic esophageal leiomyomatosis since age 9. His mother had persistent hematuria and esophageal and genital leiomyomas. ASDL-4 is a male with AS who developed cataracts and symptomatic esophageal leiomyomatosis at age 4. His mother had hematuria and esophageal leiomyomatosis. ASDL-5 had AS, hematuria, and a grossly dilated esophagus.
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 - Abbreviations for the amino acid residues are as follows: A, Ala; C, Cys; D, Asp; E, Glu; F, Phe; G, Gly; H, His; I, Ile; K, Lys; L, Leu; M, Met; N, Asn; P, Pro; Q, Gln; R, Arg; S, Ser; T, Thr; V, Val; W, Trp; and Y, Tyr.
 - We thank J. Gruen, L. Ollitervo, M. Mariyama, H. Lemmink, and L. Kluytmans for scientific discussion and technical assistance and P. Cochat and B. Roussel for referring patients. Supported by the Sigrid Juselius Foundation, the Academy of Finland, National Institutes of Health, and the Howard Hughes Medical Institute.

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Identification of a Structural Glycoprotein of an RNA Virus as a Ribonuclease

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One of the three structural glycoproteins of classical swine fever virus (CSFV) is E0, a disulfide-bonded homodimer that induces virus-neutralizing antibodies and occurs in a virion-bound as well as a secreted form. E0 was shown to be similar to a family of fungal and plant ribonucleases. Purified E0 from CSFV-infected cells was a potent ribonuclease specific for uridine and inhibitable by zinc ions.

Members of the genus *Pestivirus*, family *Flaviviridae*, are causative agents of severe animal diseases. The positive strand RNA genome of pestiviruses encodes one large polypeptide that is processed to the mature viral proteins (1, 2). The CSFV structural glycoprotein E0 forms a disulfide-bonded homodimer with an apparent molecular size of 97 kD (3). Each monomer consists of 227 amino acids that correspond to amino acids 268 to 494 of the CSFV polyprotein (2, 4). Accordingly, about one-half of the mature viral protein consists of carbohydrate. The amino acid sequence of E0 lacks a transmembrane anchor, and the glycoprotein is secreted from infected cells (4). E0 is also localized at the surface of the virion (5); linkage to the virion occurs by an as yet unknown mechanism.

Comparative sequence analysis, including extensive searches for degenerate func-

tional motifs in the deduced primary structure of E0, led to the identification of sequence features characteristic of a family of fungal and plant ribonucleases (RNases). Significant sequence similarity was shared by two regions in the E0 amino acid sequence and the most conserved regions of the extracellular RNases T2 of

Table 1. Hydrolysis of various nucleic acids by purified E0 as measured by perchloric acid precipitation (22) at 37°C. A value of 100% corresponds to 0.015 A_{260} units $\text{min}^{-1} \text{ml}^{-1}$. The relative activities determined for two E0 preparations are separated by slashes. Abbreviations: ds, double-stranded; ss, single-stranded.

Substrate	Relative activity (%)
Yeast RNA	100/100
<i>Escherichia coli</i> ribosomal RNA	51/47
<i>Escherichia coli</i> tRNA	48/53
PolyU	1755/1673
PolyA	2/2
PolyC	0/0
PolyG	0/0
ds DNA	0/0
ss DNA	0/0

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