American Journal of Pathology, Vol. 152, No. 3, March 1998
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Short Communication

Somatic Deletion of the 5′ Ends of Both the *COL4A5* and *COL4A6* Genes in a Sporadic Leiomyoma of the Esophagus

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Leiomyomata of the esophagus are sporadic benign tumors of unknown etiology. We studied a collection of nine tumors for the expression of extracellular matrix components and found the same aberrant expression pattern as previously observed in inherited diffuse leiomyomatosis. We demonstrate here the occurrence of a somatic deletion at the *COL4A5/COL4A6* locus at Xq22 in a frozen leiomyoma sample. These data confirm the hypothesis that the same underlying etiology is responsible for circumscribed smooth muscle proliferation in sporadic leiomyomata as for diffuse smooth muscle cell proliferation in inherited diffuse leiomyomatosis. *(Am J Pathol 1998, 152:673–678)*

Leiomyomata of the esophagus (EL) are sporadic benign tumors, mainly affecting adults.[1] They are characterized by the proliferation of well differentiated smooth muscle cells arranged in a plexiform pattern. They do not show any sign of malignancy, and mitoses are infrequent. No malignant degeneration is observed, although carcinomas located over the tumors, and thought to be secondary to chronic stimulation of the epithelium covering the lesion, have been reported.[2,3] Esophageal leiomyomata form circumscribed and often solitary nodules. This is in contrast to diffuse esophageal leiomyomatosis (DL) where thickened esophageal musculature exhibits extensive replacement of the normal fiber pattern by irregular, plexiform fibers with whorl formation. In DL, the proliferation is observed predominantly in the lower third but may extend variably through the rest of the esophagus and into the upper stomach. The tumor process may also involve the tracheobronchial tree and the female genital tract.

Inherited cases of DL have been reported,[4–6] and in most families, the disease is associated with X-linked Alport syndrome (AS), an inherited nephropathy due to mutations in the *COL4A5* gene, which encodes the $\alpha 5$ chain of type IV collagen.[7,8] This gene, located on the X chromosome, is arranged head-to-head with *COL4A6*, which encodes the corresponding $\alpha 6$ chain[9] and contains two alternatively used first exons named 1 and 1′.[10] We have previously shown that patients with diffuse leiomyomatosis and Alport syndrome (DL-AS) have germline deletions removing the 5′ ends of both genes, encompassing only *COL4A6* exons 1, 1′, and 2 but extending variably within *COL4A5*.[11] We also showed that both the $\alpha 5$ and $\alpha 6$ chains of type IV collagen, which are normally present in smooth muscle cell basement membranes, are absent in the tumor basement membranes.[12] In addition, we identified other abnormalities in the expression of noncollagenous components of smooth muscle cell basement membranes and of $\beta 1$ integrin subunits in the esophageal tumors of DL-AS patients.[12]

On the basis of these results and the similar smooth muscle origin of EL and DL, we hypothesized that a somatic event similar to the germline mutations observed in DL could be responsible for the development of sporadic EL. In an attempt to confirm this hypothesis, we analyzed the expression of various basement membrane

Supported by the Association Francaise contre les Myopathies, the Association pour la Recherche sur le Cancer, la Ligue Nationale contre le Cancer (LNC), and the MENESR (ACC-SV95). Y. Cai was supported by a grant from la Fondation pour la Recherche Médicale.

Accepted for publication December 1, 1997.

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Table 1. Distribution of Basement Membrane Components in Normal Esophagus and in Esophageal Leiomyoma

	Antibody	Normal esophagus	Patient F EL	Origin of the antibodies
Type IV collagens				
α1	MAB1	+++	+++	Wieslab AB, Lund, Sweden
α3	MAB3	−	−	Wieslab AB
α4	MAB85	−	−	Ref. 29
α5	MABA7	+++	−	Ref. 29
α6	MABH63	+++	−	Ref. 30
Type VI collagen	MAB5C6	+++	+++	Hybridoma Bank, Iowa City, IA
Fibronectin	AC 24911	+++	±	Institut Pasteur, Lyon, France
Nidogen	MAB A9	+++	+++	Ref. 31
Laminin chains				
α1	MAB 1924	++	++	Chemicon International, Temecula, CA
β1	MAB 1921	+++	−	Chemicon
γ1	MAB 1920	+++	+++	Chemicon
β2	MAB C4	+	+	Hybridoma Bank
Integrins				
α1	CD49a	+	+	Immunotech, Marseille, France
α2	CD49b	±	−	Immunotech
α3	CD49c	++	++	Oncogene Science, Cambridge, MA
α4	CD49d	−	−	Immunotech
α5	CD49e	+++	±	Oncogene Science
α6	CDw49f	−	−	Immunotech
α8	Rabbit anti-α8	±	±	Ref. 32
β1	Mouse anti-β1	+++	+++	Locus, Helsinki, Finland

components in nine tumors and looked for a somatic *COL4A5/COL4A6* rearrangement in a frozen sporadic EL.

Materials and Methods

Tissues

Nine surgically removed EL, from five male and four female patients, were collected for immunohistochemical studies. Eight were embedded in paraffin and one (from a female patient) was frozen immediately after removal. Normal esophageal tissue was obtained from the autopsy of a 7-year-old male child. One sample was immediately frozen and another was fixed in formaldehyde and embedded in paraffin.

Immunohistochemistry

Antibodies used in this study are described in Table 1. Affinity-column-purified fluorescein isothiocyanate (FITC)-labeled sheep IgG F(ab')2 fragment and anti-rabbit and anti-mouse immunoglobulins were from Silenus Laboratories (Hawthorn, Victoria, Australia). Immunohistochemical staining was performed on sections of the frozen tumor, and the distribution of type IV collagen chains was analyzed in all of the eight paraffin-embedded samples after pretreatment by microwave heating.[12]

DNA Analysis

Peripheral blood samples were taken, after informed consent, from the female patient whose tumor was frozen and from two controls (male and female) and were used to prepare high molecular weight DNA for pulsed-field gel electrophoresis[11] and DNA for conventional Southern blotting.[13] High molecular weight DNA from the frozen tumor was prepared as follows. Frozen tissue was ground to a powder and homogenized in phosphate-buffered saline (PBS) using a glass homogenizer. Cells were counted in a hemocytometer and resuspended at 50×10^6/ml in PBS. An equal volume of low-melting-point agarose (Sea Plaque GTG, FMC BioProducts, Rockland, ME) previously dissolved in PBS and maintained at 50°C was added to the cells. The mixture was dispensed into precooled plastic molds. Embedded cells were then treated as described previously.[11] Tumor DNA was also extracted by standard procedures.[13]

Hybridizations were performed with the *COL4A5* probe JZ4[14] and the *COL4A6* probes JZ3, JZE3, and JZK22,[15] which cover the 5′ ends of the respective cDNAs (see Figure 4). The *COL4A6* genomic probes LA226-EH[9] and B78-4E,[11] which include *COL4A6* exon 1′ and 198 bp of the intergenic region, and *COL4A6* exon 3 and its flanking intronic sequences, respectively, were also used.

Scanning densitometry was performed on Southern blots.[16] To determine the gene copy number, Southern blots were successively hybridized with the probe LA226-EH and with a non-X control probe, a 2.1-kb *Eco*RI fragment from the cosmid 23182,[17] located on the short arm of chromosome 19. Superimposed autoradiograms were analyzed by scanning densitometry (Hoefer Scientific Instruments GS300). The peak areas corresponding to each hybridization signal were calculated by electronic integration. The values for control and patient constitutional DNA and for tumor DNA were calculated through three independent determinations. Various ratios were deduced from these values. The *R* ratio was obtained by dividing the intensity of the fragment detected by the allele-specific probe LA226-HE by that of the fragment

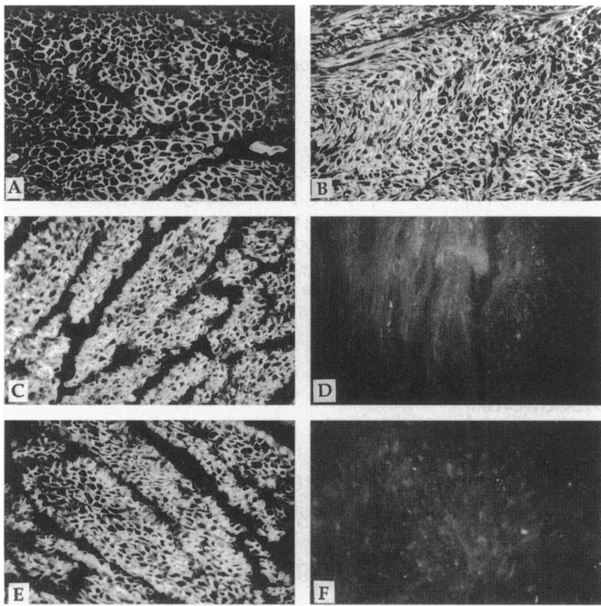

Figure 1. Staining of normal esophagus (**A**, **C**, and **E**) and tumor esophagus (**B**, **D**, and **F**) with anti-α1(IV) (**A** and **B**), anti-α5(IV) (**C** and **D**), and anti-α6(IV) (**E** and **F**) collagen antibodies.

detected by the control probe. The *P/C* ratio was obtained by dividing the *R* ratio of patient DNAs by that of the female control DNA. A *P/C* ratio around 1 indicates that the DNA studied contains two alleles of the fragment detected by LA226-EH as does the female control, whereas a *P/C* ratio around 0.5 indicates that the test DNA contains only one copy of the allele.

Results

Immunohistochemistry

Normal esophageal distribution of basement membrane proteins in control samples have been previously described.[12] The frozen EL as well as the eight paraffin-embedded EL samples were tested for type IV collagen expression (Figure 1). We also tested the frozen tumor for the expression of the main noncollagenous extracellular matrix components and β1 integrins (Figure 2). Results for frozen tumor were compared with those from normal frozen esophagus and are shown in Table 1. As in normal tissue, all nine tumors expressed both the α1(IV) collagen chain (Figure 1, A and B) and the [$\alpha1(IV)_2$-α2(IV)] molecule (data not shown). In contrast, no staining was observed with anti-α5(IV) and -α6(IV) collagen chain antibodies (Figure 1, D and F), although these two chains are shown to be expressed in smooth muscle cell basement membranes of normal frozen or paraffin-embedded esophagus (Figure 1, C and E). The study of noncollagenous components of the tumor matrix revealed additional abnormalities. Whereas nidogen was expressed in the tumor as in normal tissue (Figure 2, A and B), there was no labeling for the β1 chain of laminin (Figure 2D), and the expression of fibronectin was dramatically reduced (Figure 2F). The pattern of expression of the β1

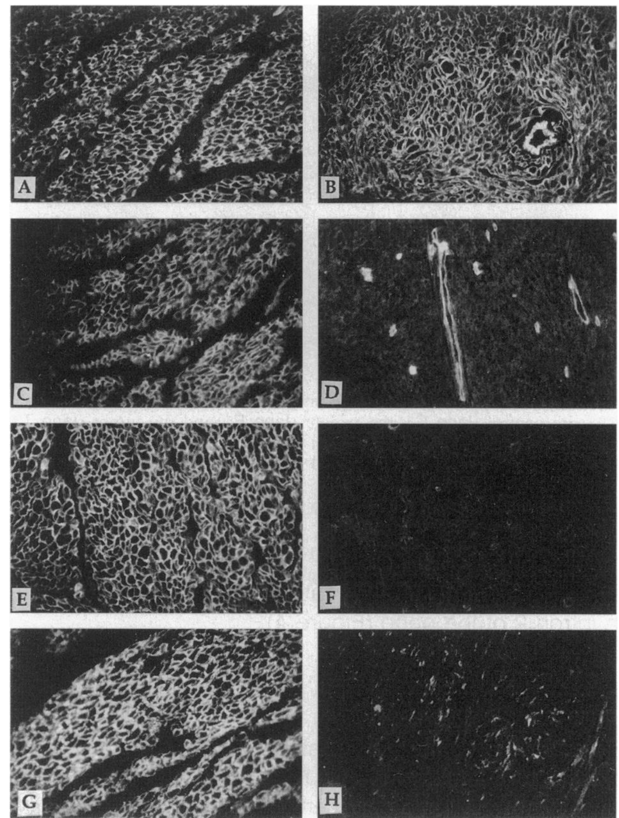

Figure 2. Staining of normal esophagus (**A**, **C**, **E**, and **G**) and tumor esophagus (**B**, **D**, **F**, and **H**) with anti-nidogen (**A** and **B**), anti-β1 laminin (**C** and **D**), anti-fibronectin (**E** and **F**), and anti-α5 integrin (**G** and **H**) antibodies.

integrin subunits showed, by comparison with normal esophagus, an irregular and faint expression of α5 integrin (Figure 2, G and H). The other α subunits and the β1 chain had patterns similar to those in the control tissue (data not shown).

Pulsed-Field Gel Electrophoresis

No differences were observed between patient and female control constitutional DNA on hybridization of *Nru*I or *Sfi*I digests with any of the *COL4A5* or *COL4A6* probes. In contrast, the tumor DNA, hybridized with the probes JZ4, JZ3, B784E, JZE3, or JZK22, detected a 520-kb fragment in addition to the normal 650-kb *Nru*I fragment (Figure 3A). This fragment did not hybridize to LA226-EH (Figure 3B), showing that it did not result from a variant sequence but was a junction fragment resulting from a deletion. As would be predicted from these data, *Sfi*I digestion of the tumor DNA showed, in addition to the normal 180- and 40-kb bands, a 55-kb junction fragment when hybridized with JZ4 (Figure 3C). This fragment was not hybridized by LA226-EH either (data not shown). Hybridization of *Sfi*I-digested tumor DNA with B78-4E gave the same pattern as control DNA. Taking into account the map of the region, these results indicate a 130-kb somatic heterozygous deletion in the tumor, removing *COL4A5* exon 1 as well as *COL4A6* exons 1, 1′,

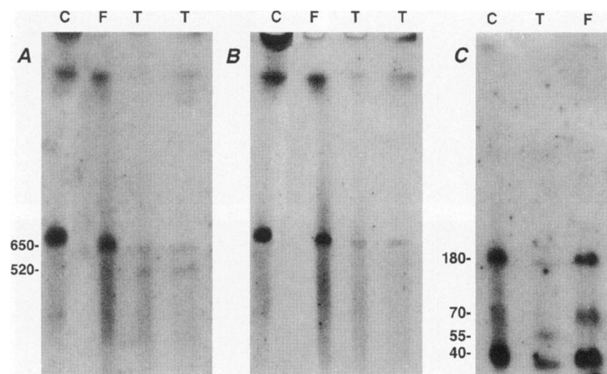

Figure 3. Autoradiograms of *Nru*I (**A** and **B**) and *Sfi*I (**C**) digested DNA from a female control (**lane C**), the patient (**lane F**), and the tumor (**lane T**), hybridized with JZ4 (**A** and **C**) and LA226-EH (**B**). The 70-kb *Sfi*I/JZ4 fragment observed in both the control and the patient is a partial digestion product.

and 2 with a breakpoint located upstream of the *Sfi*I site in intron 2 of the gene (Figure 4).

Densitometry Analysis

Southern blots of *Eco*RI digests for patient F constitutional and tumor DNA revealed in the latter a reduction of the intensity of the 1.4-kb fragment, obtained on hybridization with LA226-EH. The results of the densitometry analyses are shown in Table 2 and Figure 5. Whereas the *P/C* ratio was 0.9 in the patient's constitutional DNA, it was found to be 0.55 in the tumor, a result that indicated that the tumor carried only one copy of LA226-EH, whereas the constitutional DNA carried two. Similar conclusions were also drawn when a male DNA sample was used as a control (data not shown). The average intensity of the 1.4-kb *Eco*RI fragment hybridized to LA226EH was 60% in tumor DNA with respect to the patient's constitutional DNA. Although not statistically significant, the 10% higher-than-expected intensity in the tumor may be accounted for by contamination of the tumor by normal tissue. These results are consistent with the loss of an allele in the tumor.

Table 2. Densitometry Values Obtained by Scanning of Southern Blots of Patient Tumor, Patient Constitutional, and Female Control DNA

DNA source	LA226-EH	Autosomal control probe	*R*	*P/C*
Patient tumor	3534	6448	0.548	0.552
Patient blood	4481	4948	0.905	0.911
Female control blood	4027	4054	0.993	

The values shown here are the average of three deposits, each deposit having been independently scanned three times.

Discussion

Somatic mutations of genes involved in inherited tumors have been reported in several nonfamilial cancers[18–22] and more rarely in benign tumors, such as mutations of the neurofibromatosis type 2 (*NF2*) gene in sporadic schwannoma[23] and meningioma,[24] both tumor types arising in neurofibromatosis type 2. In most cases, somatic inactivation of a tumor suppressor gene known to be involved in an inherited tumor is observed in sporadic tumors of the same type. However, dominant activating mutations of RET have been reported both in multiple endocrine neoplasia as germline mutations and in sporadic medullary thyroid carcinoma as somatic mutations.[22]

Here we analyzed nine sporadic EL for type IV collagen chains distribution and observed in all tumors the absence of both the α5 and α6(IV) chains, as described in DL associated with AS, an inherited condition observed in patients bearing germline deletions of the *COL4A5/COL4A6* genes.

In addition, the study of the main extracellular matrix components in a frozen EL showed the absence of the β1 chain of laminin and of α5 integrin and a dramatic decrease in the expression of fibronectin. These striking features, which demonstrate that, even in a benign tumor process, significant changes in the extracellular matrix composition and cell-matrix interactions can occur, are also observed in inherited DL. Although some or all of

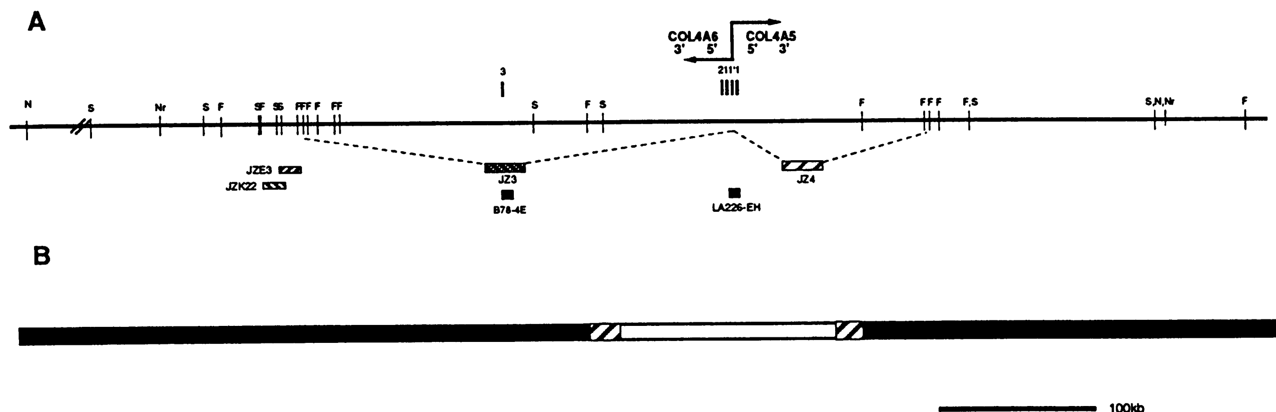

Figure 4. Long-range restriction map of the *COL4A5/COL4A6* locus[11] and schematic representation of the somatic deletion observed in the leiomyoma. **Open boxes** represent the deletion, and **hatched boxes** represent the potential breakpoint region.

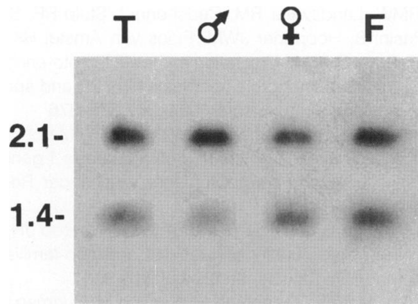

Figure 5. Example of dosage analysis of tumor (T) and constitutional (F) patient DNA and male and female control DNA. The upper and lower bands are the 2.1- and 1.4-kb fragments detected by the control probe and LA226-EH, respectively.

these modifications might be secondary phenomena related to changes in the synthesis of extracellular matrix components by proliferating cells, our observation of similar immunohistochemical changes in both sporadic leiomyomata and inherited diffuse leiomyomatosis suggested that a common mechanism could be involved in both disorders. This prompted us to look for a DNA rearrangement at the *COL4A5/COL4A6* locus in an EL frozen sample.

In this study, we report for the first time a somatic deletion of the 5′ ends of the *COL4A5* and *COL4A6* genes in a sporadic esophageal leiomyoma of a female patient with normal genotype. The deletion mimics the germline mutations consistently observed in patients with diffuse esophageal leiomyomatosis and Alport syndrome. The only genetic abnormalities reported to date in esophageal leiomyomata are cytogenetic abnormalities[25] that, contrary to uterine leiomyomata, are relatively rare, and may be a secondary phenomenon, as demonstrated in some uterine leiomyomata short-term cultures.[26] Here, the clonal origin of the tumor is highly suggested by the complete absence of staining of smooth muscle cells of the tumor with α5 and α6 type IV collagen antibodies. This suggests that the absence of these type IV collagen chains is not a secondary alteration and that the *COL4A5/ COL4A6* deletion is an early event. Furthermore, the absence of α5(IV) and α6(IV) chains in the basement membranes of eight additional EL suggests that these tumors may also bear a similar *COL4A5/COL4A6* deletion, although we cannot exclude other mechanisms that could account for the loss of expression. This could not be verified as the tumor samples had been formalin fixed and paraffin embedded, making them unsuitable for DNA extraction and therefore for analysis by PFGE or conventional analysis. None of the polymorphic markers mapping to the *COL4A5/COL4A6* region[27,28] are localized in the expected deletion (data not shown). This prevented us from searching loss of heterozygosity in the tumor samples.

The mechanism by which the *COL4A5/COL4A6* deletion could lead to the development of the tumor remains to be elucidated. Analysis of the *COL4A5/COL4A6* deletions in AS patients with and without DL showed that DL develops exclusively in patients with deletions removing only exons 1, 1′, and 2 of *COL4A6*, whereas AS patients with deletions extending further than exon 3 in *COL4A6* are consistently unaffected with DL, suggesting a dominant activating effect of the mutation in DL.[11] However, despite the obvious implication of this region in the pathogenesis of DL within the DL-AS framework, in the absence of a candidate entity, this hypothesis remains open to debate. As such, this report of a similar mutation in a sporadic and nonsyndromic condition of similar etiology greatly strengthens the case. It is of particular interest that, as in inherited DL, the breakpoint of the deletion in the sporadic leiomyoma studied here is, once again, located in *COL4A6* intron 2.

In summary, we have shown that a somatic deletion of the 5′ ends of the *COL4A5/COL4A6* genes, similar to the germline mutations found in inherited DL, occurred in a sporadic esophageal leiomyoma, suggesting that the mechanism leading to smooth muscle cell proliferation is the same in the two conditions, and ratifying the role of sequences localized in the *COL4A6* intron 2 in the development of these mesenchymal tumors.

Acknowledgments

We thank Drs. J. Zhou and E. Tournier-Lasserve for providing several *COL4A6* probes and the control probe, respectively. The monoclonal antibodies MAb5C6 against type VI collagen and MAbC4 against laminin β2, developed by E. Engvall and J. Sanes, respectively, were obtained from the Developmental Studies Hybridoma Bank maintained by the Department of Pharmacology and Molecular Sciences, Johns Hopkins University School of Medicine, Baltimore, MD, and the Department of Biology, University of Iowa, Iowa City, IA, under contract NO1-HD-2-3144 from the NICHD.

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