Genetic and morphologic determinants of pneumothorax in lymphangioleiomyomatosis

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Genetic and morphologic determinants of pneumothorax in lymphangioleiomyomatosis. Am J Physiol Lung Cell Mol Physiol 293: L800–L808, 2007. First published July 6, 2007; doi:10.1152/ajplung.00176.2007.—Lymphangioleiomyomatosis, a multisystem disease affecting women, is characterized by proliferation of abnormal smooth muscle-like cells in the lungs, leading to cystic destruction of the parenchyma and recurrent pneumothoraces. Clinical characteristics of lymphangioleiomyomatosis patients were analyzed to determine the relationship of pneumothoraces to disease progression. Patients were genotyped for polymorphisms in genes of extracellular matrix proteins collagen, elastin, and matrix metalloproteinase-1 to assess their association with pneumothoraces. Clinical data and polymorphisms in the genes for types I and III collagen, elastin, and matrix metalloproteinase-1 were compared with the prevalence of pneumothorax. Of 227 patients, 57% reported having had at least one pneumothorax. Cyst size on high-resolution computed tomography scans was associated with pneumothorax; patients with a history of pneumothorax were more likely to have larger cysts than patients who had no pneumothoraces. In patients with mild disease, those with a history of pneumothorax had a faster rate of decline in forced expiratory volume in 1 s (FEV1; P = 0.001, adjusted for age) than those without. Genotype frequencies differed between patients with and without pneumothorax for polymorphisms in the types I and III collagen, elastin, and matrix metalloproteinase-1 genes. Larger cysts may predispose lymphangioleiomyomatosis patients to pneumothorax, which, in early stages of disease, correlates with a more rapid rate of decline in FEV1. Polymorphisms in types I and III collagen and matrix metalloproteinase-1 genes may cause differences in lung extracellular matrix that result in greater susceptibility to pneumothorax.

LYMPHANGIOLEIOMYOMATOSIS (LAM), a rare multisystem disease affecting primarily women, is associated with proliferation of smooth muscle-like “LAM cells,” leading to destruction of lung parenchyma, with cyst formation, lymphatic abnormalities, and abdominal tumors (e.g., angiomyolipomas) (47). The presence of thin-walled cysts throughout the lung is a notable characteristic of LAM. LAM may occur sporadically or with tuberous sclerosis complex (TSC), an autosomal dominant neurocutaneous syndrome, associated with hamartoma formation in multiple organ systems, and caused by mutations in one of two tumor suppressor genes, TSC1 or TSC2 (11). LAM cells are characterized by the presence of mutations in a TSC gene, usually TSC2 (6, 45).

Pneumothoraces occur frequently in LAM and are one of the primary symptoms leading to diagnosis in one-third of patients (41). Recent studies found prevalence rates of pneumothoraces in LAM patients of 56% (41), 63% (10), and 69% (51). In the last study, 41% of patients with pneumothorax believed that this contributed to the decline in lung function, although no differences in oxygen usage were documented (51). The incidence of repeated spontaneous pneumothorax in LAM is greater than that found in other chronic pulmonary diseases (1).

LAM cells appear to be capable of metastatic spread, in which LAM cells travel to the lungs from elsewhere in the body (perhaps from angiomyolipomas or the lymphatic system) (23). To metastasize to the lung, the LAM cell must be able to travel in the blood/lymphatic system, penetrate the vessel wall, and invade the lung interstitium (32). In the lungs, LAM cells are thought to be responsible also for destruction of the parenchyma, leading to cyst formation (16, 21, 34). Both processes involve degradation of the extracellular matrix (ECM). By immunohistochemistry, LAM lung nodules contain matrix metalloproteinases (MMPs)-1, -2, -9, and -14, which degrade collagen, as well as areas of disrupted/degraded collagen and elastin (16, 21, 34). MMP-1 degrades fibrillar collagen, whereas MMPs-2 and -9 degrade denatured collagen or gelatin (46). Collagens I and III are the major interstitial collagens of the lung and provide the architectural framework for the alveolar wall (48). Type I collagen is a triple helix composed of two α1 chains, encoded by the COL1A1 gene, and one α2 chain, encoded by the COL1A2 gene (17, 36). Type III collagen is a homotrimer of polypeptides encoded by the COL3A1 gene (17, 36). Mutations in genes encoding ECM proteins are associated with diseases characterized by weakness of the ECM (e.g., Ehlers-Danlos, osteogenesis imperfecta) (12, 31, 36, 39), and polymorphisms in these genes have been associated with disease (8, 20, 33, 39). Pneumothorax is found in patients with Ehlers-Danlos type IV and is correlated with a reduced amount of type III collagen in the lungs (9, 38, 44), while spontaneous pneumothorax also occurs in Marfan patients, possibly due to irregular elastin fibers that have been deposited on microfibrils containing mutated fibrillin-1 (5, 29).

Here, we compared the clinical data on patients with a history of pneumothorax to those who had never had a pneumothorax, focusing on disease progression and severity. Based
on the hypothesis that the occurrence of pneumothoraces may be related to differences in genes involved in ECM formation, collagen, elastin, and MMP-1 polymorphisms were compared in LAM patients and matched healthy volunteers. Frequencies of genotypes among LAM patients, grouped by clinical phenotypes, were also examined.

MATERIALS AND METHODS

Study population. The research was approved by the Institutional Review Board of National Heart, Lung, and Blood Institute (NHLBI protocol 95-H-0186), and informed consent was obtained from all participants. LAM patients were matched to volunteers based on ethnicity, gender, and age (±5 years; NHLBI protocol 96-H-100). Of the 204 patients genotyped, 8 were Asian, 9 were African-American, 2 were Hispanic, and 185 were Caucasian. Patients responded to a questionnaire regarding history of pneumothorax or pleurodesis and were also questioned during hospital visits. Our patient population overlaps considerably the population of LAM patients in the NHLBI LAM registry study, and consistent with that, the incidence of pneumothorax in the groups is similar (57 vs. 56%) (41). One hundred eighty-four patients were in the NHLBI LAM registry study; 43 were not. Our data include information for visits for several years that were not part of the registry.

Computed tomography. High-resolution computed tomography (CT) images (2) were graded as follows: grade I <30% abnormal lung, grade II = 30–60% abnormal, grade III >60% abnormal. CT scans were also assigned scores describing the average cyst size, with size I <0.5 cm, size II = 0.5–1.0 cm, and size III >1.0 cm. The lungs were divided into three equal zones by dividing the scans into three equal subsets of images. The percentage of the area judged abnormal reflected the average of the severity of involvement with cysts in each zone. Predominant or mean cyst size was calculated similarly. CT scores were assigned by a radiologist blinded to a patient’s genotype.

Pulmonary function testing. Pulmonary function testing was only performed on stable patients; patients suffering/recovering from pneumothorax or pleurodesis were not encouraged to travel to National Institutes of Health (NIH). The yearly rate of decline in lung function was calculated as described in Statistical methods.

Genotyping. Genomic DNA was prepared from whole blood using the PureGene kit (Gentra Systems, Minneapolis, MN). Polymorphisms were genotyped as indicated in Table 1.

Table 1. Genotyping

<table>
<thead>
<tr>
<th>Polymorphism</th>
<th>Primers</th>
<th>Method of Genotyping</th>
</tr>
</thead>
<tbody>
<tr>
<td>COL1A1 SpI</td>
<td>S: 5’-TAA CTT CTT GAG TAT TTG CGG ACT TTT TGG-3’, AS: 5’-GTC CAG CCC TCA TCC TGG CC-3’</td>
<td>PCR-RFLP with MscI*</td>
</tr>
<tr>
<td>RsaI</td>
<td>S: 5’-GAA ACT CTC ACA TCC CCA TCA G-3’, AS: 5’-AGT AGA GAG ATT ATT AAA TGC A-3’</td>
<td>PCR-RFLP with RsaI</td>
</tr>
<tr>
<td>MnlI</td>
<td>S: 5’-AGA CCA AGA ATT CCG CTG CG-5’, AS: 5’-TTG GAT GCA AGG TTG AAT GCA CTT TTG G-3’</td>
<td>PCR-RFLP with MnlI</td>
</tr>
<tr>
<td>COL1A2 EcoRI</td>
<td>S: 5’-GGA TCC AAA GTC ACA CAT CTA GAG-3’, AS: 5’-CAA TTC ATA TTC TTA TCC TG-3’</td>
<td>PCR-RFLP with EcoRI</td>
</tr>
<tr>
<td>MspI</td>
<td>S: 5’-TGT AAG TTT GTG AAT ACC AGT-3’, AS: 5’-CAT CAA CTT CAT AGT CCT TGG-3’</td>
<td>PCR-RFLP with MspI</td>
</tr>
<tr>
<td>(GT)n</td>
<td>S: 5’-Hex-TGT CTA CCA CGG CTG CAT AAT TCC-3’, AS: 5’-AAT ATG AAC TAC TGG GTA AGT-3’</td>
<td>PCR followed by fragment analysis†</td>
</tr>
<tr>
<td>(ACT)n</td>
<td>S: 5’-Hex-TTG CCC AAA GTC ACA CAT CTA GAG-3’, AS: 5’-CCT ATC ATA TTA TCC TG-3’</td>
<td>PCR followed by fragment analysis</td>
</tr>
<tr>
<td>COL3A1 Exon 31</td>
<td>S: 5’-TGC TGG TGC CCC TGG TGA A-3’, AS: 5’-ACC CTG AAA ATA AGT GAG A-3’</td>
<td>PCR-RFLP with AluI</td>
</tr>
<tr>
<td>VNTR</td>
<td>S: 5’-Hex-GGC AAG AGA ATC ACT AGA AC-3’, AS: 5’-CCT CCA AGG GCA ATA AC-3’</td>
<td>PCR followed by fragment analysis</td>
</tr>
<tr>
<td>Elastin Ser{222}Gly</td>
<td>S: 5’-TAC TTG GCT CCC TCC CCT TCC C-3’, AS: 5’-CTC AGG GAA ATT GGC AAC-3’</td>
<td>PCR followed by sequencing‡</td>
</tr>
<tr>
<td>Intron 20</td>
<td>S: 5’-ATC TCC CCA GTC CTT TCC CCC CCC AAA TCC GCA-3’, AS: 5’-CTC CCA CCT CTT TCC CCC CAC TCC T-3’</td>
<td>PCR followed by sequencing</td>
</tr>
<tr>
<td>MMP1 -1607</td>
<td>S: 5’-CAG TGG CAA GTG TCC TTT GG G-3’, AS: 5’-CTC CCA CCT CTT TCC CCA CAT TA-3’</td>
<td>PCR followed by sequencing</td>
</tr>
</tbody>
</table>

*PCR-RFLP: Fragments containing the polymorphism were amplified, followed by restriction enzyme digestion. Restriction enzymes were purchased from New England Biolabs (Beverly, MA). †PCR followed by fragment analysis: Fragments containing the repeats were amplified with fluorescently labeled primers, followed by analysis on a 3100 Genetic Analyzer (Applied Biosystems, Foster City, CA). ‡PCR followed by sequencing: Fragments containing the polymorphism were amplified, followed by sequencing using the Big Dye Terminator Reaction mix and analysis on a 3100 Genetic Analyzer.

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as an additive model within the LAM population. All tests were considered significant at the 0.05 level.

RESULTS

Of 227 patients with LAM who responded to a questionnaire, 57.3% reported having had at least one pneumothorax. Of the positive responders, 14.7% had 1 pneumothorax, 55.8% had 2 to 5, 17.8% had 6 to 10, and 11.6%, more than 10 pneumothoraces. Pleurodesis was performed in 83.1% of those with a history of pneumothorax. Clinical data (age at diagnosis, age of first symptoms, rate of decline of FEV₁, rate of decline of DLCO, initial pulmonary function values, cyst size, CT grade, oxygen use, transplant history, etc.) were available for a subset of the 227 patients.

There were no significant differences between the patients with and without histories of pneumothorax, with regard to diagnosis of TSC, rates of transplantation or survival, oxygen use, initial FEV₁, DLCO, or FEV₁/FVC, or rates of decline in FEV₁ or DLCO (Table 2). Patients with a history of pneumothorax differed significantly from those without in regard to initial forced vital capacity (FVC) and total lung capacity (TLC); patients with histories of pneumothoracides had smaller initial FVC (P = 0.002) and initial TLC (P < 0.001) values than those without (Table 2). Differences in FVC and TLC may reflect whether a pleurodesis was performed: FVC and TLC were significantly different between patients with pneumothorax who had pleurodesis vs. those who did not (Table 3).

CT grade is a measure of the extent of lung involvement with LAM (percentage of the lung volume judged abnormal) as viewed on CT scans, defined as grade I <30% abnormal lung, grade II = 30–60% abnormal, and CT grade III >60% abnormal. CT grade did not differ significantly in LAM patients with and without a history of pneumothorax; although there was, however, a trend with more patients with a history of pneumothorax in CT grade I than those without a history of pneumothorax (P = 0.078; Fig. 1B). Patients with a history of pneumothorax were more likely to have size II or size III cysts than to have size I cysts (P = 0.046; Fig. 1A). As 44% of LAM patients who were cyst size I had a history of pneumothorax compared with 63% of those with cyst size III, there was substantial risk of pneumothorax even with smaller cysts.

On closer analysis of the patients in CT grade I, those with a history of pneumothorax differed significantly (P < 0.05) from those without a history in initial FEV₁, initial FVC, age, age at diagnosis, age at first symptoms, diagnosis of TSC, and rate of decline of FEV₁ (Fig. 2 and Table 4). When those variables were considered for inclusion in the multivariate model, the rates of decline in FEV₁ differed significantly between those with and those without pneumothoraces after adjusting for age and age at diagnosis; those who had had at least one pneumothorax exhibited a more rapid decline in FEV₁ [no pneumothorax: decline of 0.2 ± 0.4% predicted per year (n = 27) vs. pneumothorax: decline of 2.8 ± 0.6% predicted per year (n = 46); P = 0.001]. It seems that in patients who had incurred at least one pneumothorax, FEV₁ was declining more rapidly early in the disease process. Although patients with a history of pneumothorax were diag-

Table 2. Comparison of clinical characteristics of LAM patients with or without pneumothorax

| PFT, yr‡ | 0.9 (103) | 0.9 (102) | 0.003 |
| PFT, yr§ | 0.4 (102) | 0.3 (100) | 0.001 |
| Survival, yr | 0.4 (92) | 0.04 |

Abbreviations: TSC, tuberous sclerosis complex; LAM, lymphangioleiomyomatosis; FEV₁, forced expiratory volume in 1 s; FVC, forced vital capacity; FEV₁/FVC, ratio of FEV₁ to FVC; TLC, total lung capacity; DLCO, diffusing capacity for carbon monoxide; CT, computed tomography; NS, not significant; P, probability.
nosed earlier in their disease based on high-resolution CT, their initial FEV₁ values were surprisingly lower than those of patients with no history of pneumothorax (85.0 ± 2.3 vs. 97.4 ± 3.1%; P = 0.002); their initial FEV₁/FVC values were not, however, significantly different.

The difference in the rate of decline in FEV₁ between patients in CT grade I who had a history of pneumothorax and those without a history of pneumothorax did not appear to be affected by pleurodesis. There was a significant difference in the rate of decline in FEV₁ between those without pneumothorax and those with pneumothorax and no pleurodesis (P = 0.028) and between those without pneumothorax and those with both pneumothorax and pleurodesis (P = 0.002; Table 5). Interestingly, there was also a significant difference in the rate of decline in DLCO between those without pneumothorax and those with pneumothorax and no pleurodesis (P = 0.002; Table 5).

When a similar analysis was performed for CT grades II and III, no significant differences were found between patients with and patients without pneumothorax when comparing rates of decline in FEV₁ or in DLCO, initial FEV₁, initial DLCO, or initial FEV₁/FVC (data supplement Tables 1 and 2; the online version of this article contains supplemental data). While initial FVC and initial TLC were not different between CT grade II patients with and without pneumothorax, these measurements were significantly different for those in CT grade III, with those patients with a history of pneumothorax having smaller percent-predicted values for FVC (P = 0.005) and TLC (P = 0.001) than those without a history of pneumothorax.

To assess possible relationships/correlations with susceptibility to LAM or pneumothoraces, polymorphisms in collagen and elastin genes were investigated. As MMP-1 is one of the few MMPs capable of degrading fibrillar collagens (46), we also genotyped patients for an MMP-1 polymorphism.

Type I collagen comprises two α(1) chains and one α(2) chain (17). Three polymorphisms of the α(1) chain gene, COL1A1, were studied: 1) Sp1, replacement of a guanine (G) by thymine (T) in intron 1 in an Sp1 transcription factor-binding site (19, 33); 2) RsI, a restriction-fragment-length polymorphism (RFLP) near the 5'-end of the gene (3, 40); 3) MmII, an RFLP in the 3’-untranslated region (UTR) (40). There were no significant differences in frequencies of the genotypes between LAM patients and normal volunteers (data supplement Table 3), or between patients with and patients without histories of pneumothoraces (data not shown). Four polymorphisms in the gene for the α(2) chain, COL1A2, were examined: 1) EcoRI, an RFLP in intron 12 (3, 40); 2) MspI, an RFLP in intron 47 (3, 37); 3) GTT, a dinucleotide repeat in intron 1 (7, 40); 4) ACT, a trinucleotide repeat in intron 12 (39, 40). There were no significant differences between LAM patients and normal volunteers in the genotype frequencies of any of these polymorphisms (data supplement Table 3). The frequencies of the genotypes of the MspI polymorphism and of the alleles of the (GT)n or (ACT)n polymorphism did not differ between patients with and patients without histories of pneumothoraces (data not shown). Frequencies of the genotypes of the EcoRI polymorphism were significantly different in patients with vs. patients without pneumothoraces. More patients who had had pneumothoraces lacked the restriction site (EE: pneumothorax: 57.1%, no pneumothorax: 32.1%), whereas those without pneumothorax were more likely to have the restriction site (ee) or be heterozygous (Ee: P = 0.001; Table 6).

Type III collagen is encoded by the gene COL3A1, which has at least two polymorphisms. The exon 31 polymorphism is a replacement of G by adenine (A) that results in replacement of alanine by threonine (8, 52). The variable number tandem repeat (VNTR) polymorphism is a 15-bp repeat in intron 25 (35). No difference between LAM patients and normal volunteers was found in allele frequencies of the VNTR polymorphism (data supplement Table 3) or between patients with and patients without histories of pneumothoraces (data not shown). Frequencies of the genotypes of the ColA1 polymorphism were significantly different in patients with pneumothorax; patients without pneumothorax had more GG, and those with pneumothorax had more AG and AA (P = 0.025; Table 6). There was a trend of association of the ColA1 polymorphism with cyst size (P = 0.06). Patients with cyst size III tended to have either the AA or AG genotype (7.3 and 58.5%, respectively), whereas those with cyst size I tended to have the GG genotype (54.8%). As pneumothorax was associated with cyst sizes II and III, and the
frequencies of COL3A1 AA and AG were associated with pneumothorax, the association between the COL3A1 polymorphism and cyst size, while not reaching significant level, was supportive of the link between cyst size, type III collagen, and pneumothorax.

We examined two polymorphisms in the gene for elastin: Ser(422)Gly, resulting from replacement of A by G (24), and in intron 20, a change of cytosine (C) to T (26). Genotype frequencies for these polymorphisms did not differ between LAM patients and normal volunteers (data supplement Table 3) or between LAM patients with and patients without pneumothoraces (data not shown).

A polymorphism in the MMP-1 gene promoter at $\Delta/\Delta$ is an insertion (2G)/deletion (1G) of a guanine, with the insertion creating an ETS transcription factor-binding site (49). Genotype frequencies of the MMP-1 polymorphism were not significantly different in LAM patients and normal volunteers (data supplement Table 3); they tended to be significantly different between LAM patients with and patients without pneumothoraces, as frequency of the homozygous 2G form was higher among patients with pneumothorax, and those without pneumothorax had more 1G/1G and 1G/2G ($P = 0.052$; Table 6).

Some of the polymorphisms in the COL1A2, COL3A1, and MMP1 genes were found to be associated with pneumothorax, but none of them were associated with the development of LAM when the genotypes of the LAM patients were compared with those of healthy volunteers after age and ethnicity match-
Table 4. Comparison of clinical characteristics of LAM patients in CT grade I with or without pneumothorax

<table>
<thead>
<tr>
<th></th>
<th>No Pneumothorax</th>
<th>Pneumothorax</th>
<th>P Value†</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age at diagnosis, yr</td>
<td>41.2±1.5 (27)</td>
<td>35.4±1.2 (47)</td>
<td>0.004</td>
</tr>
<tr>
<td>Age of first symptom, yr</td>
<td>38.6±1.8 (27)</td>
<td>31.6±1.3 (47)</td>
<td>0.002</td>
</tr>
<tr>
<td>Age as of 12/2005, yr</td>
<td>48.8±1.6 (27)</td>
<td>43.6±1.1 (47)</td>
<td>0.007</td>
</tr>
<tr>
<td>PFT, yr§</td>
<td>4.8±0.2 (27)</td>
<td>5.2±0.3 (46)</td>
<td>NS</td>
</tr>
<tr>
<td>Duration, yr§</td>
<td>7.6±0.7 (27)</td>
<td>8.5±0.4 (46)</td>
<td>NS</td>
</tr>
<tr>
<td>TSC</td>
<td>15 (55.6%)</td>
<td>37 (78.7%)</td>
<td>0.034</td>
</tr>
<tr>
<td>LAM-TSC</td>
<td>12 (44.4%)</td>
<td>20 (21.3%)</td>
<td></td>
</tr>
</tbody>
</table>

*Data are shown as n, (%) for categorical variables, or means ± SE for continuous variables. †P values compare either line items (i.e., initial FEV1) in those without pneumothorax vs. those with pneumothorax) or group characteristics (i.e., percentage with or without TSC for those patients with or without a history of pneumothorax). NS indicates a P value >0.10. §PFT (yr) refers to the number of years the patients have undergone pulmonary function testing. ¶Duration (yr) is the length of time the patients have had LAM.

Table 5. Effect of pneumothorax on CT grade I LAM patients

<table>
<thead>
<tr>
<th>Pneumothorax</th>
<th>Pleurodesis</th>
<th>% Predicted</th>
<th>Rate of Decline</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>FVC</td>
<td>TLC</td>
</tr>
<tr>
<td>−</td>
<td>−</td>
<td>100.3±2.5 (26)</td>
<td>94.6±1.9 (26)</td>
</tr>
<tr>
<td>+</td>
<td>−</td>
<td>99.2±5.7 (8)</td>
<td>95.9±4.8 (8)</td>
</tr>
<tr>
<td>+</td>
<td>+</td>
<td>88.8±2.6 (38)</td>
<td>88.0±2.2 (38)</td>
</tr>
</tbody>
</table>

−, + indicate patients with or without a history of pneumothorax or pleurodesis. Data are shown as means ± SE (n). There were no CT grade I patients without pneumothorax that had a pleurodesis. *P = 0.002 for comparison of rate of decline in FEV1 in patients with no pneumothorax or pleurodesis vs. those with both pneumothorax and pleurodesis. †P = 0.028 for comparison of rate of decline in FEV1 in patients with no pneumothorax or pleurodesis vs. those with pneumothorax without pleurodesis. §P = 0.002 for comparison of rate of decline in DLCO in patients with no pneumothorax or pleurodesis vs. those with pneumothorax without pleurodesis.

DISCUSSION

Pneumothorax is the sentinel symptom for diagnosis in about one-third of LAM cases (41). In our study, 57% of the patients had experienced at least one pneumothorax, and 16 patients had 10 or more pneumothoraces; 83% of the patients with pneumothorax also had a pleurodesis. Comparison of clinical characteristics of patients with and without pneumothoraces revealed no significant differences regarding diagnosis of TSC, oxygen use, transplantation rate, survival, initial FEV1 and DLCO, initial FEV1/FVC, and rates of decline of FEV1 and DLCO. Patients with a history of pneumothorax had significantly lower values for initial FVC and TLC than those without a history of pneumothorax. Patients with cyst sizes II and III were more likely to have had a pneumothorax than those with size I (P = 0.046). Larger cysts or factors responsible for the cyst formation would lead presumably to a greater probability of rupture of the pleura. While the possibility exists that the association between cyst size and pneumothorax is really an association reflecting duration of disease (i.e., the longer the duration of disease, the more likely the patient is to have larger cysts and pneumothorax), it is difficult to accurately determine duration of disease for the LAM patients. Patients with pneumothorax tend to be diagnosed with LAM earlier than those with asthma-like symptoms, leading to
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Table 7. Genotypes of LAM patients by CT grade

<table>
<thead>
<tr>
<th>CT Grade</th>
<th>COL1A1</th>
<th>ELN</th>
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<tbody>
<tr>
<td></td>
<td>SpI</td>
<td>Ser(422)Gly</td>
</tr>
<tr>
<td></td>
<td>GG 36 (52.9)</td>
<td>5 (7.1)</td>
</tr>
<tr>
<td></td>
<td>GT 25 (36.8)</td>
<td>18 (26.0)</td>
</tr>
<tr>
<td></td>
<td>TT 7 (10.3)</td>
<td>13 (19.7)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>13 (19.7)</td>
</tr>
</tbody>
</table>

Data are shown as n (percent). P values compare genotype frequencies across CT grades.

a longer apparent duration for the pneumothorax patients purely because the disease was diagnosed earlier. Cyst size showed a statistical trend to the rate of decline in FEV1 (P = 0.006) and in DLCO (P = 0.026), with cyst size III associated with faster rates of decline in both over the entire LAM patient population (data not shown). There is a large risk of pneumothorax even for those patients with cyst size I, as 44% of these patients had a history of pneumothorax. A trend was detected suggesting that those with pneumothorax were more likely to be CT grade I, while those without pneumothorax were more likely to be CT grade III (P = 0.078). The association of CT grade with pneumothorax may reflect the fact that patients with pneumothorax may seek medical attention earlier in the disease, be diagnosed sooner, and therefore be CT grade I and early in the observed course of disease. CT grade showed significant correlation to the rate of decline in FEV1 (P < 0.001) and in DLCO (P = 0.024) over the LAM population as a whole, with CT grade III associated with faster rates of decline in both (data not shown).

There were differences among patients in CT grade I between those with and those without pneumothoraces. The former had significantly lower initial FEV1 and faster rates of decline of FEV1. Multiple regression analysis accounting for age and age at diagnosis confirmed a significantly faster rate of decline in FEV1 in those with a history of pneumothorax than those without, which was not true for patients in CT grades II and III. This conclusion does not change if patients with less than five pulmonary function measurement points are excluded from the analysis to decrease the variability of the rate of decline (data not shown). These data suggest that early in disease, pneumothorax can be indicative of a greater decline in flow rate, i.e., FEV1, but not DLCO.

This finding was unexpected as most studies show that patients with pneumothorax have a milder course of disease than those without pneumothorax (10, 22). In a study of Japanese LAM patients, the 15-yr survival rate of those with the initial symptom of pneumothorax was 89%, whereas for those with dyspnea as the first symptom, it was 47%, suggesting that patients with pneumothorax as an initial symptom progressed more slowly (22). In our study, there was no difference in survival rates of patients with and without pneumothorax (15-yr survival rate of 92.0% for no pneumothorax; 91.3% for with pneumothorax). We defined the category of pneumothorax in this study as ever having had a pneumothorax, not just as the initial symptom; this may well account for differences in conclusions of the two studies. Another study of LAM patients from the US and UK found that those with pneumothorax were significantly less likely than those without to have breathlessness at rest or dyspnea with exertion (10). In this study also, similar to ours, those with pneumothorax had significantly lower FVC and TLC values than those without, which may be a result of pleurodesis (Table 3) (10). A different study suggested, however, that 41% of patients who had had pneumothorax felt subjectively that the event had led to a decline in lung function, although this was not confirmed by changes in oxygen use, reflecting more severe disease (51). These findings would resemble ours, in which there was no difference overall in disease course between those with and without pneumothorax; the faster rate of decline was seen only in patients with milder disease and not in later stages of disease.

As the LAM cell is capable of metastasis (23) and contributes to cyst formation (16, 21, 34), we looked for associations of collagen, elastin, and MMP-1 gene polymorphisms with clinical features of LAM patients. Differences in the COL1A2, COL3A1, and MMP-1 genes were significantly associated with pneumothorax. Frequencies of the EE form of the EcoRI polymorphism in COL1A2, of the AA genotype in the exon 31 polymorphism of COL3A1, and of the 2G/2G homozygotes were higher in patients with than those without pneumothorax. These polymorphisms reflect some of the published polymorphisms found in these genes; other unstudied variants in these genes may be important.

Polymorphisms of COL1A1 and elastin were also significantly associated with CT grade. These and polymorphisms in COL1A2 and COL3A1 have been associated with differences in bone mineral density (COL1A1, COL1A2) (19, 30, 33, 39), risk of floppy mitral valve/mitral valve prolapse (COL3A1) (8), and differences in carotid artery distensibility (elastin) (20), but only the SpI polymorphism of COL1A1 has been shown to have functional impact (33). The SpI polymorphism introduces an SpI site in intron 1 of the gene when the T allele is present, leading to an increase in transcription (19, 33). Subjects with the TT genotype have less bone mineral density than those heterozygous for the polymorphism, while those who are GG homozygotes have the highest bone mineral density (33). GT heterozygotes have an increased ratio of A1(I) chain relative to A2(I) chain, which may lead to changes in bone quality (33).

Mutations in COL1A1 and COL1A2 have been found in patients with osteogenesis imperfecta, osteoporosis, and Ehlers-Danlos syndrome (EDS) type VIIA and VIIB (12, 13, 30, 31, 36); mutations in COL3A1 are responsible for EDS type IV (31). Type IV is the most severe of the EDS syndromes, with an increased chance of premature death due to rupture of major arteries or the gastrointestinal tract (31). These patients have reduced amounts of type III collagen in their lungs and other tissues (9). Although pneumothorax is not a common clinical manifestation of the other types of EDS, recurrent pneumothoraces do occur in patients with EDS type IV (9, 14, 24, 38, 43, 44), consistent with a link between pneumothorax and reduced amounts of type III collagen. We report here also an association between a COL3A1 polymorphism and pneumothorax in LAM patients. Pneumothorax also occurs in patients with Marfan syndrome (29), possibly due to irregular elastin fibers that are deposited on microfibrils containing mutated fibril-
MMP-1, i.e., a genetic predisposition to pneumothorax, cyst thorax and degradation of type I and III collagens by COL3A1, and MMP-1 genes were associated with pneumothoraces. The findings that polymorphisms in COL1A2, greater in LAM patients with than those without pneumothorax, increased risk of pneumothorax and there was a trend toward association of a type III collagen polymorphism with larger cyst size. We were unable to evaluate an interaction between the two genes in the risk of pneumothorax, as there were no COL3A1 AA plus MMP-1 2G/2G individuals in the study group. It is possible that an increase in MMP-1 (resulting from the 2G form of the MMP-1 promoter) leads to more degradation of the A form of the type III collagen, larger cysts, and increased risk of pneumothorax.

In addition to MMP-1, MMP-2 and MMP-9 are found in LAM nodules. A recent report (4) shows that MMP-9 is able to cleave collagen types I and III in their soluble, monomeric forms. It may be interesting to examine the roles of these MMPs in pneumothorax.

Failure to correct for multiple comparisons appropriately is a major cause of false positive results in genetic association studies. Our study only focused on a dozen of polymorphisms in several important candidate genes but did not test a large number of random markers. Therefore, use of Bonferroni correction may result in serious false negatives. We also tested several clinical characteristics, most of which were correlated. Again, it would be overly conservative and cause false negative results if the Bonferroni correction were used for highly correlated characteristics. Therefore, our study was not corrected for multiple comparisons. As expected for this type of study, these results will need to be replicated or alternate proof gathered to definitively state COL1A2, COL3A1, and MMP1 have a role in pneumothorax.

Spontaneous recurrent pneumothorax occurs with great frequency in LAM patients. We found that cyst size was correlated with pneumothorax, larger cysts with greater numbers of pneumothoraces. In addition, in early disease (those in CT grade I), the rate of decline in FEV1 was greater in LAM patients with than those without pneumothorax. The findings that polymorphisms in COL1A2, COL3A1, and MMP-1 genes were associated with pneumothorax suggest that there may be a link between pneumothorax and degradation of type I and III collagens by MMP-1, i.e., a genetic predisposition to pneumothorax, cyst size, and rate of decline in lung function.

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