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Evidence That Lymphangiomyomatosis Is Caused by *TSC2* Mutations: Chromosome 16p13 Loss of Heterozygosity in Angiomyolipomas and Lymph Nodes from Women with Lymphangiomyomatosis

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Summary

Lymphangiomyomatosis (LAM) is a rare disease, of unknown etiology, affecting women almost exclusively. Lung transplantation is the only consistently effective therapy for LAM. Microscopically, LAM consists of a diffuse proliferation of smooth muscle cells. LAM can occur without evidence of other disease (referred to as "sporadic LAM") or in association with tuberous sclerosis complex (TSC). TSC is an autosomal dominant tumor suppressor gene syndrome characterized by seizures, mental retardation, and tumors in the brain, heart, skin, and kidney. Renal angiomyolipomas occur in ~50% of sporadic LAM patients and in 70% of TSC patients. Loss of heterozygosity (LOH) in the chromosomal region for the *TSC2* gene occurs in 60% of TSC-associated angiomyolipomas. Because of the similar pulmonary and renal manifestations of TSC and sporadic LAM, we hypothesized that LAM and TSC have a common genetic basis. We analyzed renal angiomyolipomas, from 13 women with sporadic LAM, for LOH in the regions of the *TSC1* (chromosome 9q34) and *TSC2* (chromosome 16p13) genes. *TSC2* LOH was detected in seven (54%) of the angiomyolipomas. We also found *TSC2* LOH in four lymph nodes from a woman with retroperitoneal LAM. No *TSC1* LOH was found. Our findings indicate that the *TSC2* gene may be involved in the pathogenesis of sporadic LAM. However, genetic transmission of LAM has not been reported. Women with LAM may have low-penetrance germ-line *TSC2* mutations, or they may be mosaic, with *TSC2* mutations in the lung and the kidney but not in other organs.

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Introduction

Lymphangiomyomatosis (LAM), which affects women almost exclusively, is a rare disease of unknown etiology that was first described ~60 years ago (Van Stossel 1937). The average age at onset of symptoms, which include shortness of breath (67%), lung collapse (25%), coughing (12%), and chest pain (10%), is 33 years (Taylor et al. 1990; Johnson et al. 1993). Chest x-rays typically reveal a diffuse interstitial infiltrate, with no predominance in any one lung zone. Although most LAM is pulmonary, cases with retroperitoneal, pelvic, or perirenal involvement in lymph nodes and extranodal sites have been reported (Torres et al. 1995). LAM usually is diagnosed by open lung biopsy. Microscopically, there is cystic distortion of the normal pulmonary architecture by an atypical smooth muscle infiltrate (fig. 1). Most patients have a slowly declining clinical course (Taylor et al. 1990). Lung transplantation is the only effective therapy for patients with end-stage disease (Boehler et al. 1996; Kalassian et al. 1997).

LAM is diagnosed in ~1/1,000,000 people, in both Europe and the United States. The actual incidence is likely to be higher, however, since LAM is difficult to distinguish clinically from other pulmonary diseases and is often misdiagnosed as asthma, chronic obstructive pulmonary disease, or bronchitis (Taylor et al. 1990).

LAM can occur as an isolated disorder (here referred to as "sporadic LAM") or in association with tuberous sclerosis complex (TSC). TSC is an autosomal dominant disorder characterized by seizures, mental retardation, and hamartomatous tumors of the brain, heart, kidney, lung, and skin. These tumors include cerebral cortical tubers, cardiac rhabdomyomas, renal angiomyolipomas, LAM, and facial angiofibromas. LAM affects 4.6% of women with TSC (Castro et al. 1995), an incidence that is ~20,000 × that of the general population. Among patients with TSC, LAM is the third-most-frequent cause of TSC-related death, after renal disease and brain tumors (Castro et al. 1995).

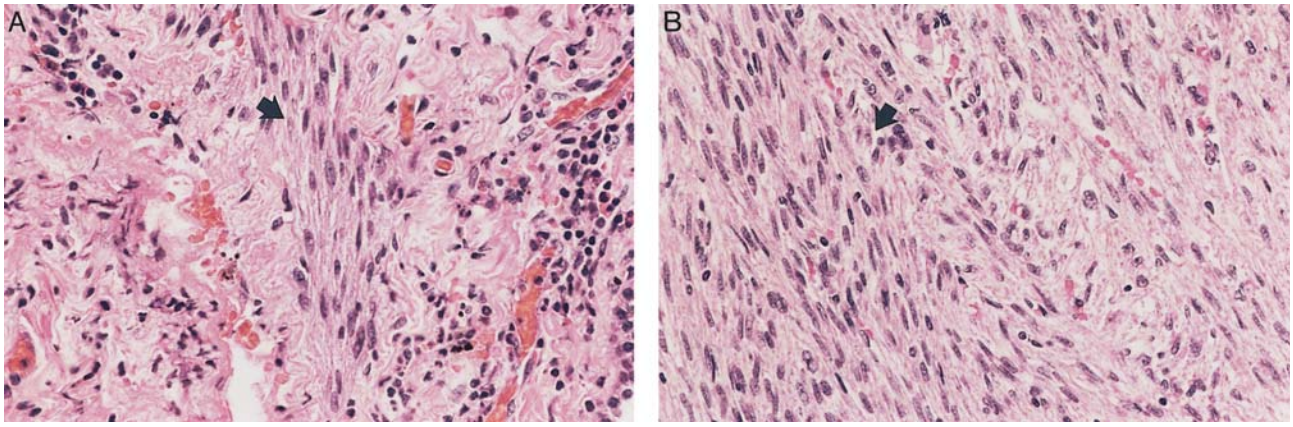

Figure 1 Histopathological similarity between the smooth muscle cells of LAM (*left*) and of angiomyolipomas (*right*). Arrows indicate regions of smooth muscle proliferation. (Stained with hematoxylin and eosin; original magnification × 40.)

Angiomyolipomas are benign tumors consisting of fat, smooth muscle (fig. 1), and vascular elements. Renal angiomyolipomas occur in two-thirds of patients with TSC (Bjornsson et al., in press) and in ~50% of women with sporadic LAM (Kerr et al. 1993; Bernstein et al. 1995; Maziak et al. 1996). In contrast to the angiomyolipomas associated with TSC, which are usually multiple and bilateral, angiomyolipomas associated with sporadic LAM tend to be solitary and asymptomatic.

Two genes are associated with TSC: *TSC2* on chromosome 16p13 (European Chromosome 16 Tuberous Sclerosis Consortium 1993) and *TSC1* on chromosome 9q34 (Van Slegtenhorst et al. 1997). Patients with TSC2 disease appear to have a higher risk of mental retardation than do those with TSC1 disease (Jones et al. 1997). Of the patients with a family history of TSC, 50% show genetic linkage to *TSC1* and 50% to *TSC2* (Povey et al. 1994). However, two-thirds of TSC patients have no prior family history of the disease and are believed to represent new mutations. Germ-line *TSC1* and *TSC2* mutations appear to be inactivating (European Chromosome 16 Tuberous Sclerosis Consortium 1993; Van Slegtenhorst et al. 1997), and loss of heterozygosity (LOH) occurs in TSC tumors (Carbonara et al. 1994; Green et al. 1994*a*, 1994*b*; Henske et al. 1995, 1996), suggesting that *TSC1* and *TSC2* are tumor suppressor genes.

Because women with TSC and those with sporadic LAM have similar lung and renal disease, we hypothesized that women with sporadic LAM have mutations in the *TSC1* or the *TSC2* gene, despite the absence of CNS, dermatologic, or other manifestations of TSC. We analyzed 13 LAM-associated angiomyolipomas and five lymph nodes containing LAM, for LOH in the chromosomal regions of the *TSC1* and *TSC2* genes.

Material and Methods

This study was approved by the Institutional Review Board of the Fox Chase Cancer Center. All patients had typical manifestations of pulmonary LAM (dyspnea, pneumothoraces, and/or chylous effusions), except for patient 500, who had extensive abdominal and retroperitoneal LAM. Five nodes from this patient were selected for analysis. One node (L1) contained lymphocytes along with smooth muscle cells. The other four nodes (L2–L5) contained almost exclusively smooth muscle cells. For the angiomyolipomas, DNA was extracted from unstained paraffin-embedded tissue in 50 mM KCl, 10 mM Tris (pH 8.3), 1.5 mM $MgCl_2$, 100 μg BSA/ml, 0.45% Tween 20, 0.45% Nonidet P-40, and 100 μg proteinase K/ml. A 2-μl aliquot of DNA was used in a 20-μl PCR reaction. Control genomic DNA was obtained from histologically normal tissue. For the LAM-associated lymph nodes, a random 15-nucleotide preamplification step was performed before the specific PCR reaction (Zhang et al. 1992). For the preamplification, DNA was purified by use of phenol-chloroform extraction and was resuspended in 10 μl water. The DNA was amplified by use of a random primer (Operon Technologies) at a 2-μM final concentration in a total volume of 100 μl, for 50 cycles consisting of 94°C for 1 min, 37°C for 2 min, a 37–55°C ramp at 10 s/°, and 55°C for 4 min, with a final extension at 72°C for 5 min. For the specific amplifications, we used three microsatellite markers on chromosome 9q34 that are within 500 kb of *TSC1—D9S149, D9S1198,* and *D9S1199* (Henske et al. 1993, 1996)—and four markers on chromosome 16p13 that are within 600 kb of *TSC2—Kg8, D16S525, D16S283,* and *D16S291* (Shen et al. 1994; Snarey et al. 1994). PCR was performed

Table 1

Results of LOH Analyses and Clinical Examinations of Eyes, Skin, and Brain for Evidence of TSC, for 14 Women with LAM

Patient	*TSC2* LOH[a]	*TSC1* LOH[a]	Clinical Evidence of TSC[b]		
			Eye	Skin	Brain CT/MRI
367	+	−		−	Normal
423	−	−		−	Normal
432	+	−	−	−	Normal
436	+	−	−	−	Normal
437	+	−		−	Normal
443	−	−	−	−	Normal
480	−	−		−	Normal
481	−	−		−	Normal
487	+	−		−	Not performed
489	−	−	−	−	Normal
490	+	−		−	Not performed
491	−	−		−	Normal
492	+	−	−	−	Normal
500	+	−		−	Normal

[a] A plus sign (+) indicates presence of LOH, and a minus sign (−) indicates absence of LOH.

[b] A minus sign (−) indicates that a specific examination for retinal hamartomas ("Eye") or for dermatologic manifestations ("Skin") was performed, and no evidence of TSC was detected.

with [^{32}P]dGTP in the reaction mix. PCR products were resolved by denaturing 8-M urea-polyacrylamide gel electrophoresis and were visualized by autoradiography. LOH was determined by visual inspection of the relative intensities of the bands, with comparison to the pattern in normal DNA from the same individual (Henske and Kwiatkowski 1998). All results were repeated at least twice for confirmation.

Results

Seven (54%) of the 13 angiomyolipomas showed LOH in the *TSC2* region of chromosome 16p13 (table 1). Representative examples of chromosome 16p13 LOH are shown in figure 2. No LOH was detected in the *TSC1* region of chromosome 9q34. The markers typed for each tumor and the results of the LOH analysis are given in table 2. Four retroperitoneal lymph nodes containing LAM, from patient 500, showed *TSC2* LOH (fig. 3). To control for the specificity of the lymph-node LOH results, which were obtained by a preamplification using a random primer, multiple normal DNA samples from patient 500 were amplified by use of the preamplification step. No evidence of allelic loss was seen. As an additional control, the preamplification step, using multiple chromosome 16p13 and 9q34 markers, was used on eight of the angiomyolipoma DNA samples, with results that were identical to those obtained without preamplification.

No patient in this study had a family history of either TSC or LAM, and no patient had any clinical signs or symptoms of TSC. The degree to which individual patients were screened for TSC by dermatologic examinations, ophthalmologic examinations, or brain imaging is given in table 1. For all patients, except patients 487 and 490, brain computed tomography (CT) or magnetic-resonance imaging (MRI) scans were performed to exclude a diagnosis of TSC. As indicated in table 1, some patients also had ophthalmologic examinations that showed no evidence of retinal hamartomas.

Discussion

LAM is a rare, nonfamilial, progressive lung disease affecting women. The distinctive histological pattern of diffuse smooth muscle proliferation and cystic degeneration of the lung interstitium also is seen in ~5% of women with TSC. Patients with sporadic LAM do not have the neurological, ocular, or dermatologic manifestations of TSC but frequently have renal angiomyolipomas. These similarities have led to speculation that LAM and TSC have a common pathogenic basis (Corrin et al. 1975; Bonetti and Chiodera 1996).

Here we report, for the first time, that LOH in the region of *TSC2* occurs in LAM-associated renal angio-

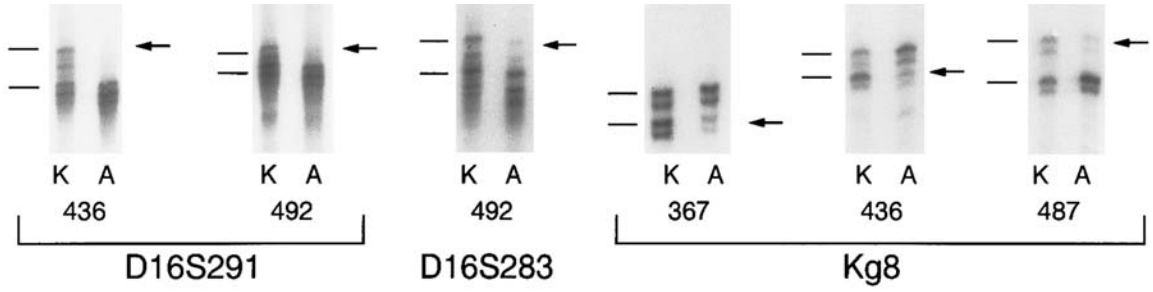

Figure 2 Examples of LOH at chromosome 16p13 markers *D16S291*, *D16S283*, and *Kg8*, in angiomyolipomas from patients 367, 436, 487, and 492. K = normal kidney; and A = angiomyolipoma. Horizontal lines indicate the upper band of each allele in the normal kidney. Arrows indicate the "lost" alleles in the angiomyolipomas.

Table 2

LOH Results for Individual Markers on Chromosomes 16p13 and 9q34

Patient	Tissue[a]	*D16S283*	*D16S291*	*Kg8*	*D16S525*	*D9S149*	*D9S1199*	*D9S1198*
367	AML	…	2	LOH	LOH	2	2	…
423	AML	1	2	1	1	…	2	…
432	AML	…	LOH	LOH	LOH	2	…	…
436	AML	1	LOH	LOH	LOH	2	1	2
437	AML	1	LOH	LOH	1	2	1	2
443	AML	1	2	1	2	2	2	2
480	AML	1	2	1	2	2	2	1
481	AML	1	2	1	1	2	2	…
487	AML	1	1	LOH	1	…	…	1
489	AML	1	2	1	2	1	2	…
490	AML	…	…	1	LOH	2	2	…
491	AML	…	1	1	2	2	…	…
492	AML	LOH	LOH	1	LOH	2	…	1
500	L1	2	2	1	2	2	…	…
	L2	LOH	LOH	1	LOH	2	…	…
	L3	LOH	LOH	1	LOH	2	…	…
	L4	LOH	LOH	1	LOH	2	…	…
	L5	LOH	LOH	1	LOH	2	…	…

NOTE.—1 = homozygous marker; and 2 = heterozygous marker without LOH. An ellipsis (…) indicates that the marker was not typed for that patient. The markers are listed in centromeric-to-telomeric order; the *TSC2* gene is between markers *Kg8* and *D16S525*, and the *TSC1* gene is between markers *D9S149* and *D9S1199*.

[a] AML = angiomyolipoma.

myolipomas and in lymph nodes containing LAM. We detected LOH in 7 (54%) of 13 angiomyolipomas from women with LAM and in four lymph nodes from one female patient with retroperitoneal LAM. In previous work, we found that 56% of TSC-associated angiomyolipomas show LOH at either the *TSC1* or the *TSC2* locus (Henske et al. 1996), compared with 10% of sporadic angiomyolipomas (i.e., those not associated with TSC or LAM) (Henske et al. 1995). The difference between the incidence of *TSC2* LOH in LAM-associated angiomyolipomas and the incidence of LOH in sporadic angiomyolipomas is statistically significant ($P < .001$, by use of Fisher's exact test). We and others have found that benign angiomyolipomas have a low incidence of genomic instability and random LOH (Carbonara et al. 1996; Henske et al. 1996).

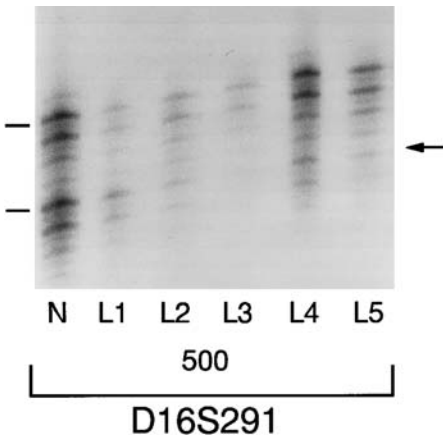

Figure 3 LOH at chromosome 16p13 marker *D16S291*, in retroperitoneal lymph nodes (L1–L5) from patient 500. Each allele is represented by a ladder of three bands. Horizontal lines indicate the upper band of each allele in normal DNA (N). Heterozygosity is retained in L1. The arrow indicates the "lost" lower alleles in L2–L5.

The *TSC2* LOH in the angiomyolipomas from women with LAM is consistent with a *TSC2* mutation in the remaining allele. In TSC (Carbonara et al. 1994) and in other germ-line tumor suppressor gene disorders (Collins et al. 1995; Cornelis et al. 1995), the LOH pattern has been consistent with loss of the wild-type allele. In sporadic tumors of many types, LOH has been predictive of somatic mutation in the remaining allele. For example, *MEN1* mutations were found in 7 of 13 sporadic parathyroid tumors with chromosome 11q13 LOH but in 0 of 20 tumors without 11q13 LOH (Heppner et al. 1997). Because LOH patterns are predictive of alleles containing somatic mutations, LOH also has been used to augment or complement linkage data from pedigrees with inherited cancer-predisposition syndromes (Lustbader et al. 1995; Hemminki et al. 1997; Rohde et al. 1997).

LOH was found only on chromosome 16p13 (*TSC2*) in the LAM-associated angiomyolipomas. This may indicate that *TSC2* mutations are more likely to cause sporadic LAM than are *TSC1* mutations. Alternatively, angiomyolipomas resulting from *TSC2* mutations may be more likely to require surgical removal, either because

they are larger than angiomyolipomas resulting from *TSC1* mutations or because they are more likely to bleed.

It is not known whether Knudson's two-hit tumor suppressor gene model (Knudson 1971) applies to the pulmonary smooth muscle cells as well as to the angiomyolipomas of LAM patients. Because of the intermingling of smooth muscle cells with normal lung parenchyma, it is difficult to isolate a pure population of pulmonary LAM smooth muscle cells for LOH analysis. However, a common pathogenic basis for the abnormal cellular proliferations in the lung (pulmonary LAM) and the kidney (angiomyolipomas) is likely. The smooth muscle cells of LAM and of angiomyolipomas appear to be closely related on the basis of histological (fig. 1), immunohistochemical (Chan et al. 1993), and ultrastructural (Peyrol et al. 1992; Kaiserling et al. 1994) criteria. In addition, the LOH in LAM-containing lymph nodes from patient 500 indicates that the two-hit model applies to extrarenal as well as to renal smooth muscle cells in LAM. The LOH pattern in the four nodes with LOH was identical (table 2), and the node without LOH contained some contaminating normal lymphocytes. Therefore, we were unable to determine whether the smooth muscle cells in the separate lymph nodes resulted from a single second-hit event or from multiple second-hit events.

Our data support a role for *TSC2* mutations in the pathogenesis of angiomyolipomas in women with LAM. This could indicate that both LAM and TSC are caused by germ-line *TSC2* mutations and are part of the same disease spectrum. The fact that genetic transmission of LAM from mother to daughter has not been reported argues against germ-line *TSC2* mutations in women with LAM. If, however, these mutations have low expression or require the interaction of specific environmental factors, such as hormonal factors, to result in smooth muscle growth, it is possible that a low frequency of genetic transmission of LAM has not yet been recognized. An alternative explanation for our findings is that LAM patients are mosaic (Hall 1988), with inactivating *TSC2* mutations in the lung and, in some cases, in the kidney but not in skin, brain, or germ-line tissue. Cases with apparent somatic mosaicism have been identified for TSC (Verhoef et al. 1995) as well as for other tumor suppressor gene syndromes (Evans et al. 1997; Lohmann et al. 1997; Sippel et al. 1997).

In summary, we found *TSC2* LOH in angiomyolipomas from seven women with sporadic LAM and in retroperitoneal lymph nodes from an eighth woman. This is the first molecular data supporting a common pathogenic basis for LAM and TSC. Since genetic transmission of LAM has not been reported, it is possible that LAM patients have germ-line *TSC2* mutations with limited expression or that they are mosaic for *TSC2* mutations.

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