Phenotypic Characterization of Disseminated Cells with *TSC2* Loss of Heterozygosity in Patients with Lymphangioleiomyomatosis

Xiong Cai[1], Gustavo Pacheco-Rodriguez[1], Qing-Yuan Fan[1]*, Mary Haughey[1], Leigh Samsel[2], Souheil El-Chemaly[1], Hai-Ping Wu[1], J. Philip McCoy[2], Wendy K. Steagall[1], Jing-Ping Lin[3], Thomas N. Darling[4], and Joel Moss[1]

[1]Cardiovascular and Pulmonary Branch, [2]Flow Cytometry Core Facility, and [3]Office of Biostatistics Research, Division of Cardiovascular Science, National Heart, Lung, and Blood Institute, National Institutes of Health, Bethesda, Maryland; and [4]Department of Dermatology, Uniformed Services University of the Health Sciences, Bethesda, Maryland

***Rationale*: Lymphangioleiomyomatosis (LAM), occurring sporadically (S-LAM) or in patients with tuberous sclerosis complex (TSC), results from abnormal proliferation of LAM cells exhibiting mutations or loss of heterozygosity (LOH) of the TSC genes, *TSC1* or *TSC2*.**

***Objectives*: To identify molecular markers useful for isolating LAM cells from body fluids and determine the frequency of *TSC1* or *TSC2* LOH.**

***Methods*: Candidate cell surface markers were identified using gene microarray analysis of human *TSC2*$^{-/-}$ cells. Cells from bronchoalveolar lavage fluid (BALF), urine, chylous effusions, and blood were sorted based on reactivity with antibodies against these proteins (e.g., CD9, CD44v6) and analyzed for LOH using *TSC1*- and *TSC2*-related microsatellite markers and single nucleotide polymorphisms in the *TSC2* gene.**

***Measurements and Main Results*: CD44v6^{+}CD9^{+} cells from BALF, urine, and chyle showed *TSC2* LOH in 80%, 69%, and 50% of patient samples, respectively. LAM cells with *TSC2* LOH were detected in more than 90% of blood samples. LAM cells from different body fluids of the same patients showed, in most cases, identical LOH patterns, that is, loss of alleles at the same microsatellite loci. In a few patients with S-LAM, LAM cells from different body fluids differed in LOH patterns. No patients with S-LAM with *TSC1* LOH were identified, suggesting that *TSC2* abnormalities are responsible for the vast majority of S-LAM cases and that *TSC1*-disease may be subclinical.**

***Conclusions*: Our data support a common genetic origin of LAM cells in most patients with S-LAM, consistent with a metastatic model. In some cases, however, there was evidence for genetic heterogeneity between LAM cells in different sites or within a site.**

Keywords: lymphangioleiomyomatosis; metastasis; loss of heterozygosity; CD9; CD44v6

AT A GLANCE COMMENTARY

Scientific Knowledge on the Subject

The multisystem manifestations of lymphangioleiomyomatosis (LAM) are believed to result from a metastatic spread of LAM cells that appear to contain mutations or loss of heterozygosity (LOH) in one of two tuberous sclerosis complex (TSC) tumor suppressor genes, *TSC1* or *TSC2*. LAM cells, however, have not been phenotypically and genetically well characterized, which will be important for their identification in clinical samples.

What This Study Adds to the Field

Here we show that specific cell surface molecules, CD9 and CD44v6, enable identification and isolation of disseminated LAM cells from patients with S-LAM, which appears to be primarily a *TSC2*-mediated disease. We report also the presence of LAM cells in bronchoalveolar lavage fluid (BALF). Patterns of *TSC2* LOH in LAM cells from different sites support a common genetic origin of LAM cells in most patients with S-LAM, but suggest also genetic and phenotypic heterogeneity of LAM cells at different sites or within a site in some cases of S-LAM.

Lymphangioleiomyomatosis (LAM) is a rare multisystem disease affecting primarily women, characterized by abnormal proliferation of smooth muscle–like LAM cells, which leads to cystic destruction of the lungs, formation of fluid-filled cystic structures in the axial lymphatics (e.g., lymphangioleiomyomas), and renal angiomyolipomas (AMLs) (1–5). LAM occurs as a sporadic disease (S-LAM) or in association with tuberous sclerosis complex (TSC) (6–8). TSC is an autosomal dominant syndrome characterized by multiorgan hamartomas, resulting from mutations in one of two tumor suppressor genes, *TSC1* on chromosome 9 (9q34) (9) and *TSC2* on chromosome 16 (16p13.3) (10, 11). LAM cells in S-LAM were reported to be associated with *TSC2* loss of heterozygosity (LOH) (12–15), consistent with Knudson's "two-hit" hypothesis (16).

LAM cells from lung nodules, AMLs, and lymph nodes of the same patient showed identical *TSC2* mutations and LOH patterns (13–15), consistent with metastatic spread among organs. Further supporting this model, LAM cells were identified in donor lungs after transplantation (17, 18) and could be isolated from blood, urine, and chyle of patients with LAM (19, 20), consistent with LAM cell dissemination in body fluids. Identification of LAM cells in blood by LOH was aided by fluorescence-activated cell sorting (FACS) removal of non-LAM cells after immunostaining with antibodies against leukocyte common antigen (CD45) and glycophorin A (CD235a) (19), a protein present on LAM cells in lung nodules. In our previous study (19), we were able to isolate LAM cells from only approximately 60% of patients, and thus could not answer questions such as whether sporadic LAM was primarily *TSC2* driven, whether LAM cells in different body fluids showed similar LOH patterns, or whether

(*Received in original form March 20, 2010; accepted in final form July 16, 2010*)

* Current affiliation: Department of Neuroscience, Cleveland Clinic, Cleveland, Ohio.

Supported in part by the Intramural Research Program of the National Institutes of Health, National Heart, Lung, and Blood Institute (J.M.); and by RO1 CA100907 (T.N.D.).

Correspondence and requests for reprints should be addressed to Joel Moss, M.D., Ph.D., Translational Medicine Branch, National Heart, Lung, and Blood Institute, National Institutes of Health, Building 10, Room 6D05, MSC-1590, Bethesda, MD 20892-1590. E-mail: mossj@nhlbi.nih.gov

This article has an online supplement, which is accessible from this issue's table of contents at www.atsjournals.org

Am J Respir Crit Care Med Vol 182. pp 1410–1418, 2010
Originally Published in Press as DOI: 10.1164/rccm.201003-0489OC on July 16, 2010
Internet address: www.atsjournals.org

LAM cells could be isolated from bronchoalveolar lavage fluid (BALF).

These questions prompted us to identify cell surface molecules unique to *TSC2*$^{-/-}$cells and use these findings to isolate LAM cells with *TSC2* LOH from BALF, urine, chyle, and blood. We have shown that CD44v6 is expressed *in situ* by LAM cells in lung nodules and is present on LAM cells grown from explanted lungs (21). This splice variant of the hyaluronic acid receptor is believed to be involved in tumor metastasis and progression (22–24). In the present study, we showed that the tetraspanin CD9, a highly expressed gene identified by microarray analysis of *TSC2*$^{-/-}$ cells from TSC skin lesions (25), and CD44v6 identified LAM cells with *TSC2* LOH from BALF, urine, and chylous effusions. Similarly, CD45^{-}CD235a^{-} and CD45^{-}CD235a^{+} cells with *TSC2* LOH were detected in blood cell fractions. The majority of *TSC2* LOH patterns were identical in LAM cells from blood, urine, and BALF or chyle from the same patients. Different LOH patterns, however, were identified in LAM cells from different body fluids in a minority of patients with S-LAM. Furthermore, we failed to find *TSC1* LOH in patients with S-LAM. Some of the results of these studies have been previously reported in the form of an abstract (26).

METHODS

Supplemental description of methods is available in the online supplement.

Patients and Sample Collection

Samples were collected from randomly selected patients with LAM (45 S-LAM and 10 TSC-LAM) and 13 healthy female volunteers who were enrolled between 2007 and 2009 at the National Institutes of Health Clinical Center in clinical protocols (95-H-0186, 96-H-0100) approved by the National Heart, Lung, and Blood Institute Institutional Review Board. The diagnosis of LAM was based on clinical, radiologic, and/or histopathologic findings.

Isolation of Cells from TSC Skin Biopsies

Fibroblasts (*TSC2*$^{+/-}$) from postauricular normal-appearing skin and fibroblast-like cells (*TSC2*$^{-/-}$) from periungual fibromas of toes from the same female patients with TSC enrolled in protocol 00-H-0051 were isolated and grown as described (25).

Immunofluorescence Analysis of Cultured Cells by Confocal Microscopy

As reported, cells were incubated with a mouse monoclonal antibody against CD9 (1:20; BD Biosciences, San Jose, CA) at 4°C for approximately 12 hours and then with the immunofluorescent fluorescein isothiocyanate (FITC)-labeled goat antibody against mouse IgG (1:100 dilution; Vector Laboratories, Burlingame, CA) for 1 hour at room temperature (21).

Fluorescence-activated Cell Sorting

Anti–CD44v6-FITC (clone VFF-7) and anti–CD9-R-phycoerythrin (PE) (clone MM2/57) antibodies were purchased from Invitrogen (Carlsbad, CA). Anti–CD9-FITC (clone M-L13), anti–CD44-R-PE (clone G44-26), anti–CD45-FITC (clone HI30), and anti–CD235a-PE (clone GA-R2) antibodies were from BD Biosciences. Cells from blood, urine, BALF, and chylous effusions were labeled for flow cytometric analysis and sorting by incubation for 30 minutes at room temperature with the indicated antibodies, followed by two washes with PBS and sorting in a MoFlo Flow Cytometer (Beckman Coulter, Inc., Fullerton, CA). Fluorescence signals were collected using amplifiers that reported on a logarithmic scale. Data acquisition, analysis, and compensation were performed using Summit software (Beckman Coulter).

Polymerase Chain Reaction Analysis of LOH

Genomic DNA was isolated from whole blood and unsorted or sorted cells using the QIAamp DNA Micro Kit (QIAGEN, Valencia, CA) and amplified (19) to determine LOH. Briefly, genomic DNA sequences were amplified at loci D16S291, Kg8, D16S3395, D16S3024, and D16S521 on chromosome 16p13.3, and at loci D9S149, D9S1198, and D9S66 on chromosome 9q34. Primer sequences were obtained from the UniSTS Database (www.ncbi.nlm.nih.gov/unists). Antisense primers were labeled with 6-FAM (Invitrogen). Polymerase chain reaction (PCR) products were analyzed on a 3100 Genetic Analyzer (Applied Biosystems, Foster City, CA). Q^{LOH} was calculated as described (19). Q^{LOH} values of less than 0.5 or more than 0.62 were scored as LOH or retention of heterozygosity (ROH), respectively, whereas no definite decision was made with Q^{LOH} values of 0.5 to 0.62 (19, 27).

Single-Nucleotide Polymorphism Analysis

Genomic DNA isolated from whole blood and unsorted or sorted cells was amplified by PCR for the exon 40 polymorphism (T5202C; rs1748) (28, 29) or for a splice site polymorphism (C482–3T; rs1800720) (28, 30), using these primer sequences: TSC40S-Hex, 5′-Hex-ATGGAG GGCCTTGTGGACAC-3′, and TSC40AS, 5′-CGGAGCCGCTTGA TGTG-3′; TSCspliceS, 5′-GGAGATGTAGATTCGGCGTC-3′, and TSCspliceAS-Hex, 5′-Hex-CTGCGGAGCTGAACTTAGG-3′. PCR products were digested with the appropriate restriction enzyme (EcoRV for T5151C; PvuII for C482–3T) (New England Biolabs, Beverly, MA), and then analyzed with a 3100 Genetic Analyzer (Applied Biosystems).

Statistical Analysis

Fisher exact test was performed with the SPSS 15.0 (SPSS, Inc., Chicago, IL). Statistical significance was accepted for $P < 0.05$.

RESULTS

We had first detected LAM cells with *TSC2* LOH in blood by OncoQuick density-gradient fractionation, and from urine and chyle specimens based on centrifugation. To improve yield and purity of LAM cells we focused on identification of potential LAM cell surface markers by comparing gene expression in TSC fibroblasts (*TSC2*$^{+/-}$) grown from normal-appearing skin and in fibroblastic cells (*TSC2*$^{-/-}$) grown from TSC-associated skin tumors of the same patient. By microarray analysis, levels of CD9 were higher in *TSC2*$^{-/-}$ cells than in their *TSC2*$^{+/-}$ counterparts (25). We reported previously that LAM cells grown from lungs contained CD44v6, a splice variant of the hyaluronic acid receptor CD44 (21), prompting us to use antibodies to these proteins to isolate circulating LAM cells.

CD9 Expression on *TSC2*$^{-/-}$ Cells

Amounts of CD9 assessed by flow cytometric analysis and immunostaining were greater in *TSC2*$^{-/-}$ skin tumor cells than in *TSC2*$^{+/-}$ skin fibroblasts (Figure 1). As determined by mean fluorescence intensity (MFI), the levels of CD9 were much higher in *TSC2*$^{-/-}$ (MFI = 71.81) than in *TSC2*$^{+/-}$ cells (MFI = 5.22) (Figure 1A). Most of the CD9 appeared to be concentrated at the plasma membrane, with a small amount located within the cells (Figure 1B). There appeared to be more intracellular CD9 in the null than in the heterozygous cells. Altogether, these data prompted us to isolate cells from body fluids based in part on the presence of this cell surface antigen.

Detection of *TSC2* LOH in CD44v6^{+}/CD9^{+} Cells from BALF and Urine

Cells from BALF, urine, and chylous effusion were incubated with anti-CD9 and anti-CD44v6 antibodies and separated by cell sorting. We found that the percentage of cells reactive with anti-CD44v6 and anti-CD9 antibodies differed considerably among body fluids and among patients, and ranged from 0.51 to 6.13% in BALF samples (n = 12), 0.12 to 8.25% in urine samples (n = 55), and 0.10 to 0.55% in chylous effusions (n =

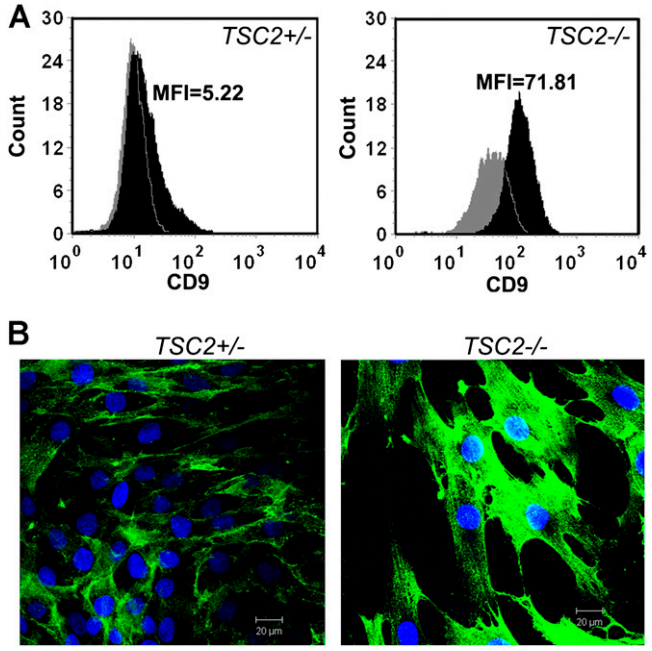


Figure 1. CD9 protein in *TSC2*$^{-/-}$ fibroblastic cells grown from human tuberous sclerosis complex (TSC)-associated skin tumors. (*A*) Flow cytometric analysis showed significantly higher levels of CD9 (clone M-L13, *black histogram*) as assessed by mean fluorescence intensity (MFI) in *TSC2*$^{-/-}$ cells (MFI = 71.81) than in *TSC2*$^{+/-}$ cells (MFI = 5.22). *Gray histogram*, negative control. (*B*) Immunostaining analysis of *TSC2*$^{+/-}$ and *TSC2*$^{-/-}$ skin cells with anti-CD9 antibody. Reactivity was greater in *TSC2*$^{-/-}$ than in *TSC2*$^{+/-}$ cells. *Blue*, nuclear staining (DAPI). Bar, 20 μm. Experiments were replicated three times.

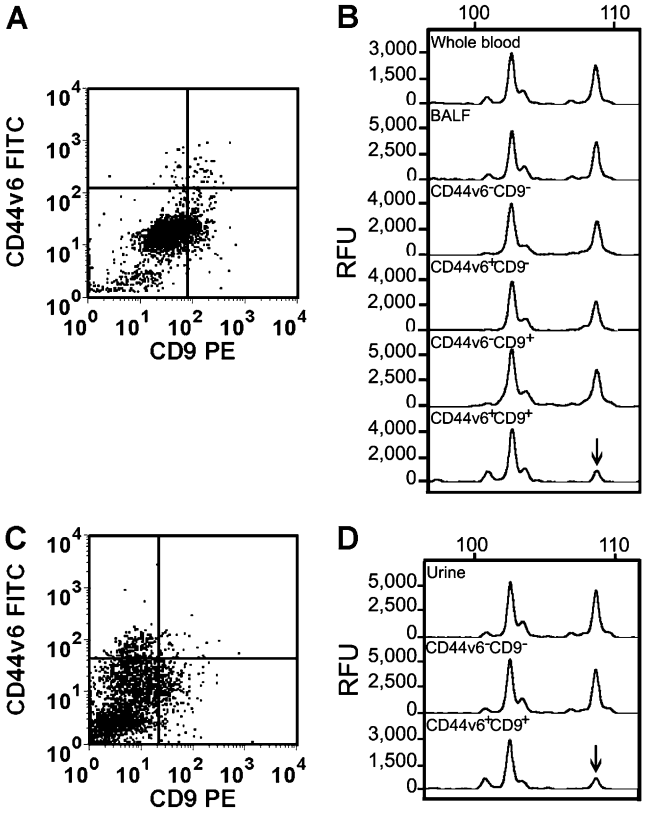


Figure 2. Fluorescence-activated cell sorting (FACS) of cells in bronchoalveolar lavage fluid (BALF) and urine samples from a patient with lymphangioleiomyomatosis (LAM). Cells were reacted with anti–CD44v6-FITC and anti–CD9-PE antibodies and sorted cells were analyzed for *TSC2* loss of heterozygosity (LOH) of chromosome 16p13.3 microsatellite marker Kg8. Four populations of BALF cells were separated (*A*), and LOH was detected only in the CD44v6$^+$CD9$^+$ population (*B, lowest histogram*). Two populations of urine cells were separated: CD44v6$^-$CD9$^-$ and CD44v6$^+$CD9$^+$ (*C*). LOH was observed only in the CD44v6$^+$CD9$^+$ population (*D, lowest histogram*). *Arrows* indicate positions of allelic loss compared with that seen in whole blood from the same patient. Numbers at the top of histograms indicate the number of DNA bases and the Y-axis indicates relative fluorescence units (RFU).

5). Genomic DNA from these cells was isolated to determine *TSC2* LOH using microsatellites near the *TSC2* locus (i.e., D16S291 [~ 331 kb centromeric to *TSC2*], Kg8 [~ 100 bp centromeric to *TSC2*], D16S3395 [~ 96 kb telomeric to *TSC2*], D16S3024 [~ 443 kb telomeric to *TSC2*], and D16S521 [~ 981 kb telomeric to *TSC2*]). We specified that a cell population had *TSC2* LOH when at least one of the informative markers (heterozygosity of the alleles) had a Q^{LOH} of less than 0.5 (*see* METHODS). Figures 2A and 2C show representative FACS plots of BALF and urine cell samples from a single patient, respectively, which were incubated with anti-CD44v6 and anti-CD9 antibodies. The CD44v6$^+$CD9$^+$ cell population from BALF showed *TSC2* LOH at the Kg8 microsatellite (Figure 2B, *bottom panel*), as did a CD44v6$^+$CD9$^+$ cell population from urine (Figure 2D, *bottom panel*). Furthermore, we were able to determine that CD44v6$^+$CD9$^+$ cells from chylous effusions had *TSC2* LOH (Table 1). These data suggest that LAM cells could be identified in BALF, urine, and chyle based on the expression of the tetraspanin CD9 and the splice variant of the hyaluronic receptor, CD44v6.

We had reported *TSC2* LOH in cells grown from LAM lung nodules and sorted with anti-CD44 and anti-CD44v6 antibodies (21), but LOH was not consistently seen in cells similarly separated from BALF (1 out of 6) and urine (2 out of 15) (*see* Figure E1 in the online supplement). Populations reactive with anti-CD45/CD235a antibodies were not seen in BALF and urine cell samples (Figure E2), nor were those reactive with anti-CD44v6/CD44 and anti-CD44v6/CD9 antibodies seen in blood cell fractions (Figure E3). Thus, LAM cells in different locations appear to show differences in surface protein expression, which is consistent with their phenotypic heterogeneity in different tissues. LAM cells in lung nodules appear both spindle-shaped and epithelioid, whereas *TSC2*$^{-/-}$ cells in renal AMLs may resemble adipocytes, vasculature, or smooth muscle cells.

In our series of 55 patients (Table E1), detection of *TSC2* LOH in blood fractions, urine, and BALF was markedly enhanced after cell sorting with the specified cell surface markers (Table 1 and Figure 3). Despite the ability to show allelic imbalance and LOH in unsorted cells from urine and blood fractions, we failed to find *TSC2* LOH in cell pellets isolated from BALF (Figure 3 and Table 1). Percentage of detection of *TSC2* LOH, however, was increased from 34% (18 of 53 patients) in unsorted blood samples separated using the OncoQuick density-gradient system to 90% (47 of 52 patients) after sorting with anti-CD45 and anti-CD235a antibodies ($P < 0.001$). Cells from urine with *TSC2* LOH were more readily detected after sorting with anti-CD44v6 and anti-CD9 antibodies (from 13% [7 of 53 patients] to 69% [36 of 52 patients]; $P < 0.001$). Furthermore, the success rate of detection of *TSC2* LOH was 70% (7 of 10 patients) in the CD44v6$^+$CD9$^+$ cell population separated from BALF samples ($P = 0.003$).

To assess the reproducibility of detection of cells with *TSC2* LOH, blood and urine samples from 10 patients with S-LAM at two visits, separated by 6 to 18 months, were analyzed and found

TABLE 1. DETECTION OF *TSC2*-RELATED LOSS OF HETEROZYGOSITY IN DIFFERENT CELL POPULATIONS FROM DIFFERENT BODY FLUIDS OF PATIENTS WITH LYMPHANGIOLEIOMYOMATOSIS

Type of LAM	Fluid	Cell Population	Number of Informative Samples*	% Samples with LOH†
S-LAM	Blood fraction	Unsorted	43	35
		$CD45^-/CD235a^-$	39	85
		$CD45^-/CD235a^+$	36	86
	Urine‡	Unsorted	43	14
		$CD44v6^-/CD9^-$	42	0
		$CD44v6^+/CD9^+$	42	67
	BALF	Unsorted	8	0
		$CD44v6^-/CD9^-$	8	0
		$CD44v6^+/CD9^-$	8	0
		$CD44v6^-/CD9^+$	8	0
		$CD44v6^+/CD9^+$	8	63
	Chyle	Unsorted	4	0
		$CD44v6^-/CD9^-$	4	0
		$CD44v6^+/CD9^+$	4	50
TSC-LAM	Blood fraction	Unsorted	10	30
		$CD45^-/CD235a^-$	9	89
		$CD45^-/CD235a^+$	9	100
	Urine‡	Unsorted	10	10
		$CD44v6^-/CD9^-$	10	0
		$CD44v6^+/CD9^+$	10	80
	BALF	Unsorted	2	0
		$CD44v6^-/CD9^-$	2	0
		$CD44v6^+/CD9^-$	2	0
		$CD44v6^-/CD9^+$	2	0
		$CD44v6^+/CD9^+$	2	100

Definition of abbreviations: BALF = bronchoalveolar lavage fluid; LAM = pulmonary lymphangioleiomyomatosis; LOH = loss of heterozygosity; PCR = polymerase chain reaction; S-LAM = sporadic LAM; TSC = tuberous sclerosis complex.

* Samples were collected from 45 patients with S-LAM and 10 patients with TSC-LAM, but only samples that were heterozygous for the markers tested and that were amplified well by PCR were included.

† Results of PCR assays are based on total of five microsatellite markers on chromosome 16p13.3: D16S291, Kg8, D16S3395, D16S3024, and D16S521.

‡ Due to limited cell numbers in urine specimens, simultaneous sorting of four cell populations led to too few cells collected for each population to be analyzed for *TSC2* LOH. We randomly selected five cases that showed *TSC2* LOH in $CD44v6^+CD9^+$ cells from urine to separate subsequently $CD44v6^+CD9^-$ and $CD44v6^-CD9^+$ cells, but did not identify *TSC2* LOH in these populations.

to show reproducible detection of *TSC2* LOH using the same microsatellites in 100% of blood samples and 80% of urine samples (Table 2 and Figure E4).

TSC2 LOH Patterns in LAM Cells from Different Body Fluids

Because the microsatellite markers on chromosome 16p13.3 near the *TSC2* gene cover a large region, we compared the patterns of *TSC2* LOH in samples from different sources in the same patients. Twenty-seven of 47 patients showed LOH at one informative microsatellite, and 20 of 47 patients at two informative microsatellites, which involved different regions of chromosome 16 (Table E1). We found that $CD44v6^+CD9^+$ cells from BALF, urine, and chylous effusions and $CD45^-CD235a^-$ or $CD45^-CD235a^+$ cells from blood fractions showed, in general, identical *TSC2* LOH patterns, that is, loss or retention of the alleles at the same microsatellite loci (Table E1). Exceptions were observed, however, in eight cases of S-LAM and two of TSC-LAM, in which LAM cells from different body fluids did not show consistent LOH or ROH for each informative marker (Table E1, *see* S-LAM2, 11, 23, 27, 30, 40, 41, 45, and TSC-LAM5, 8). In addition, we observed that allelic loss in LAM cells from different body fluids consistently occurred at the same alleles for the same microsatellite markers (Figures 2B and 2D).

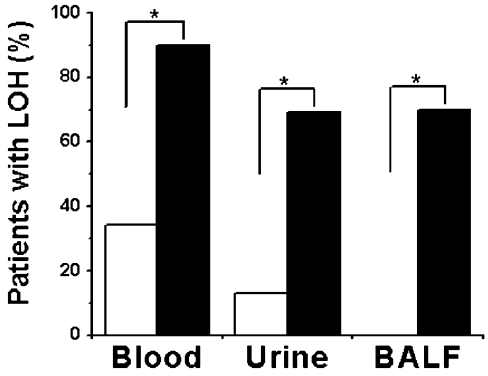


Figure 3. Effect of sorting on detection of *TSC2* loss of heterozygosity (LOH) in cell samples. Cells separated from blood by OncoQuick density-gradient centrifugation were reacted with anti–CD45-FITC and anti–CD235a-PE antibodies and cells from urine and bronchoalveolar lavage fluid (BALF) with anti–CD44v6-FITC and anti–CD9-PE antibodies. Percentage *TSC2* LOH detected in cell samples of sorted cells (*solid bars*) was significantly higher than that in unsorted cells (*open bars*) from low-density cell fractions of blood, urine, and BALF from patients with lymphangioleiomyomatosis (LAM). For all comparisons, $*P < 0.001$ for blood (n = 52) and urine (n = 52); $*P = 0.003$ for BALF (n = 10).

We analyzed also the frequency of detection of LOH with different chromosome 16p13.3 microsatellite markers (Table 3). The *TSC2* gene is closest to the Kg8 microsatellite; other markers are more distant (D16S3395 < D16S291 < D16S3024 < D16S521) and span a large region from approximately 981 kb telomeric through approximately 331 kb centromeric to the *TSC2* gene. Percentage of LOH detection by PCR using Kg8 in informative patients approached 97% in blood (n = 34), 71% in urine (n = 35), and 100% in BALF (n = 5), whereas percentage for other markers were significantly lower ($P < 0.05$). Isolated LOH was observed both centromeric and telomeric to the *TSC2* gene.

FACS of Samples from Patients with LAM and Healthy Volunteers with Anti-CD44v6 and Anti-CD9 Antibodies

Investigating whether identification of $CD44v6^+CD9^+$ cells with *TSC2* LOH would distinguish patients with LAM (S-LAM or TSC-LAM) from healthy volunteers, we found that using similar cell fractionation and sorting, cells reactive with anti-CD44v6 and anti-CD9 antibodies were also seen in BALF and urine samples from healthy volunteers. We did not observe significant differences in reactivity to anti-CD44v6 and anti-CD9 antibodies among cells from S-LAM, TSC-LAM, and healthy volunteers. Table 4 presents data for patients with S-LAM (45 blood and urine, 10 BALF, and 5 chylous effusions), patients with TSC-LAM (10 blood and urine, and 2 BALF), and healthy volunteers (13 blood, urine, and BALF). Of importance regarding the specificity of *TSC2* LOH, LOH was not detected in blood, urine, and BALF specimens from healthy volunteers (data not shown). We observed *TSC2* LOH in 38 of 43 (88%) blood specimens, 28 of 42 (67%) urine specimens, 5 of 8 (63%) BALF specimens, and 2 of 4 (50%) chylous effusions from informative patients with S-LAM. In patients with TSC-LAM, LOH was detected in 9 of 9 (100%) blood specimens, 8 of 10 (80%) urine specimens, and 2 of 2 (100%) BALF specimens. The frequency of detection of *TSC2* LOH was greater in patients with TSC-LAM than in patients with S-LAM, but it was not statistically significant. The overall detection rate in patients with LAM was 90% in blood, 69% in urine, and 70% in BALF. *TSC2* LOH was not found in five informative patients with S-LAM; two patients with S-LAM were noninformative

TABLE 2. *TSC2*-RELATED LOSS OF HETEROZYGOSITY IN BLOOD AND URINE FROM PATIENTS ON TWO VISITS

				Allelic Status of *TSC2*-Related Microsatellites				
Case no.	Visit	Interval (mo)	Fluid	D16S291	Kg8	D16S3395	D16S3024	D16S521
S-LAM1	1		Blood	NA	ROH	LOH	NI	LOH
		17	Urine	NA	ROH	ROH		ROH
	2		Blood	NA	ROH	LOH		LOH
			Urine	NA	ROH	ROH		ROH
S-LAM2	1		Blood	NA	NI	LOH	NA	NI
		18	Urine	NA		ROH	NA	
	2		Blood	NA		LOH	NA	
			Urine	NA		ROH	LOH	
S-LAM8	1		Blood	NI	LOH	LOH	NI	NI
		12	Urine		LOH	NA		
	2		Blood		LOH	NA		
			Urine		LOH	ROH		
S-LAM9	1		Blood	NI	NA	NI	NA	LOH
		18	Urine		ROH		ROH	LOH
	2		Blood		NA		NA	LOH
			Urine		ROH		ROH	LOH
S-LAM27	1		Blood	NI	LOH	LOH	NI	NI
		6	Urine		LOH	ROH		
	2		Blood		LOH	LOH		
			Urine		ROH	ROH		
S-LAM28	1		Blood	NI	LOH	NA	NI	NI
		12	Urine		LOH	ROH		
	2		Blood		LOH	NA		
			Urine		LOH	ROH		
S-LAM30	1		Blood	NI	LOH	LOH	NA	NI
		12	Urine		LOH	ROH	NA	
	2		Blood		LOH	LOH	NA	
			Urine		LOH	ROH	NA	
S-LAM31	1		Blood	NI	NI	LOH	LOH	LOH
		12	Urine			LOH	LOH	LOH
	2		Blood			LOH	NA	NA
			Urine			LOH	NA	LOH
S-LAM40	1		Blood	NI	LOH	LOH	NI	NA
		14	Urine		ROH	ROH		LOH
	2		Blood		LOH	NA		NA
			Urine		ROH	NA		NA
S-LAM43	1		Blood	NI	LOH	NI	NI	NI
		13	Urine		LOH			
	2		Blood		LOH			
			Urine		ROH			

Definition of abbreviations: LOH = loss of heterozygosity; NA = not amplified; NI = noninformative: homozygosity of the markers tested; ROH = retention of heterozygosity; S-LAM = sporadic lymphangioleiomyomatosis.

due to homozygosity of all five tested markers. We further analyzed these patients using two single-nucleotide polymorphisms (SNPs) within the *TSC2* gene. Three patients with S-LAM (S-LAM15, 35, 39) were informative for one of two SNPs. LOH at the exon 40 polymorphism was detected in $CD44v6^{+}CD9^{+}$ cells from BALF of S-LAM39 (data not shown), increasing the overall detection rate to 80% in BALF samples.

Because LAM is believed to result from mutations in the *TSC1* or *TSC2* gene, we looked to see if those lacking *TSC2* LOH had *TSC1* LOH. We therefore assessed microsatellite markers at the *TSC1* locus, but none of these samples showed *TSC1* LOH (Table E2). In addition, patients with *TSC2* ROH in blood consistently showed ROH in urine and/or BALF.

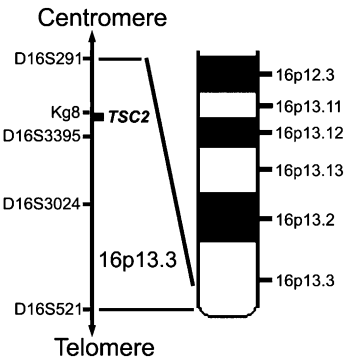
TSC2 LOH and Clinical Phenotypes of Patients with LAM

LAM is characterized by renal AMLs, lymphatic abnormalities, and pulmonary cystic lesions. We therefore assessed the association between the presence of AMLs and lymphatic involvement, which may represent the presence of more metastatic cells, and detection of LOH in cells from blood and urine. LOH was detected in 86% (n = 28) of urine samples from patients with AMLs, but in only 50% (n = 24) of urine samples from those without AMLs ($P = 0.007$), although the frequency of *TSC2* LOH was not significantly higher in blood cell fractions from patients with LAM with AMLs (n = 29) than in those without AMLs (n = 24; $P = 0.08$) (Figure 4). These data suggest that LAM cells in blood might be shed into the urine in patients with LAM, or that necrosis in the AMLs might result in $TSC2^{-/-}$ cells in the urine. There was no significant association between the presence of lymphangioleiomyomas, adenopathy, or lymphangioleiomyomas/adenopathy in patients with LAM with or without AMLs and detection of *TSC2* LOH in blood and urine cell fractions (Figure E5).

DISCUSSION

We have identified two cell surface proteins, CD44v6 and CD9, that are useful for isolation of disseminated LAM cells from BALF, urine, and chylous effusions. LAM cells from patients with S-LAM exhibit mutations and LOH most frequently in the *TSC2* locus. For the majority of patients with S-LAM, LAM cells isolated from blood, urine, BALF, or chyle of the same patients show identical *TSC2* LOH patterns for specific microsatellites, although in some, LAM cells from different body fluids appeared to differ in the extent of *TSC2* LOH regions, consistent with genetic heterogeneity.

TABLE 3. FREQUENCY OF DETECTION OF *TSC2*-RELATED LOSS OF HETEROZYGOSITY BY POLYMERASE CHAIN REACTION AMPLIFICATION OF DIFFERENT MICROSATELLITES ON CHROMOSOME 16P13.3

Marker	Positions of *TSC2* and Markers on Chromosome 16p13.3*	Distance to *TSC2*	Fluid	Number of Cases†	Number of Informative Cases‡	% with LOH in Informative Cases
D16S291		~331 kb	Blood	51	5	40
			Urine	51	6	0
			BALF	12	3	0
Kg8		~100 bp	Blood	55	34	97
			Urine	55	35	71
			BALF	12	5	100
D16S3395		~96 kb	Blood	49	32	59
			Urine	49	32	28
			BALF	11	8	13
D163024		~443 kb	Blood	53	7	29
			Urine	53	13	38
			BALF	12	4	25
D16S521		~981 kb	Blood	51	11	73
			Urine	51	17	35
			BALF	12	4	25

Definition of abbreviations: BALF = bronchoalveolar lavage fluid; LAM = lymphangioleiomyomatosis; S-LAM = sporadic LAM; TSC = tuberous sclerosis complex.

* Relative positions of microsatellite markers to the *TSC2* gene are shown.

† LAM cases included patients with S-LAM and TSC-LAM.

‡ Samples that were heterozygous for the markers tested and that were amplified well by polymerase chain reaction.

In general, these findings support the hypothesis that multisystem manifestations of LAM appear to result from a metastatic process (31). Previously in five patients with S-LAM, identical *TSC2* mutations or LOH were identified in pulmonary and renal LAM lesions (13–15). LAM cells from the recipient were identified in a transplanted donor lung (17, 18). As LAM cells were also detected in blood (19) or chylous fluids (19, 20), it was hypothesized that LAM cells could migrate or metastasize via blood and/or lymphatic circulations (19, 20). The LAM cells found in BALF could result from release from the LAM lung nodules or perhaps shedding from the lymphatic circulation within the nodules. Patients with LAM may experience chyloptysis. LAM cells were identified in chyle and BALF may contain components of chyle.

Identification of proteins on the surface of circulating or disseminated cells has been of interest in human cancers for use potentially as therapeutic targets (32–34). Gene expression microarray analysis revealed that *TSC2*$^{-/-}$ cells grown from TSC-associated skin tumors contained highly increased mRNA levels of the tetraspanin CD9 (25). We demonstrated here using flow cytometric analysis and immunostaining that CD9 protein was abundant on *TSC2*$^{-/-}$ skin tumor cells (Figure 1). A correlation between greater CD9 content and potential for metastasis is evident in some tumor types (e.g., bone, cervix, head and neck, stomach) (35), although CD9 is considered to suppress metastasis by decreasing cell motility (35–38). Tetraspanins are present widely among mammals and play important roles in cell morphology, motility, invasion, adhesion, and signaling (39–42). Tetraspanins form complexes with other tetraspanins and a variety of transmembrane proteins at tetraspanin-enriched membrane microdomains (42, 43). The diverse actions of CD9 are probably due to its association with other molecules in the tetraspanin-enriched membrane microdomains. In our studies, high levels of CD9 protein correlated with cells having *TSC2* LOH (Figure 1).

Our group had reported earlier an association between the presence of CD44v6 protein and *TSC2* LOH in LAM cells grown

TABLE 4. DETECTION OF *TSC2*-RELATED LOSS OF HETEROGEITY IN CELL SAMPLES SORTED FROM BODY FLUIDS FROM PATIENTS WITH SPORADIC-LYMPHANGIOLEIOMYOMATOSIS AND TUBEROUS SCLEROSIS COMPLEX–LYMPHANGIOLEIOMYOMATOSIS, AND FROM HEALTHY VOLUNTEERS

Group	Fluid*	Number of Cases	Noninformative†	Not Amplified	Informative‡		% Patients with LOH	
					ROH	LOH	All Cases	Informative Cases
S-LAM	Blood	45	2	0	5	38	84	88
	Urine	45	2	1	14	28	62	67
	BALF	10	2	0	3	5	50	63
	Chyle	5	0	1	2	2	40	50
TSC-LAM	Blood	10	0	1	0	9	90	100
	Urine	10	0	0	2	8	80	80
	BALF	2	0	0	0	2	100	100
Healthy volunteers	Blood	13	0	2	11	0	0	0
	Urine	13	0	1	12	0	0	0
	BALF	13	0	0	13	0	0	0

Definition of abbreviations: BALF = bronchoalveolar lavage fluid; LAM = pulmonary lymphangioleiomyomatosis; LOH = loss of heterozygosity; PCR = polymerase chain reaction; ROH = retention of heterozygosity; S-LAM = sporadic LAM; TSC = tuberous sclerosis complex.

* Cells separated from blood by OncoQuick density-gradient centrifugation were reacted with anti–CD45-FITC and anti–CD235a-PE antibodies and cells from urine, BALF, and chylous effusions with anti–CD44v6-FITC and anti–CD9-PE antibodies.

† Noninformative: homozygosity of the markers tested.

‡ Results of PCR assays are based on a total of five microsatellite markers on chromosome 16p13.3: D16S291, Kg8, D16S3395, D16S3024, and D16S521.

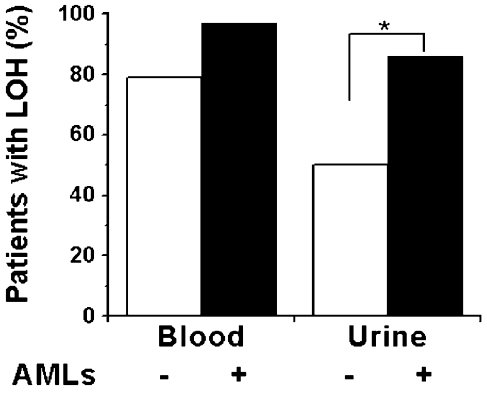


Figure 4. Association of *TSC2* loss of heterozygosity (LOH) with angiomyolipomas (AMLs). Presence of AMLs is indicated with *solid bars*, and their absence with *open bars*. LOH was detected more frequently in blood and urine from patients with lymphangioleiomyomatosis (LAM) with AMLs (blood, n = 29; urine, n = 28) than those without AMLs (blood, n = 24; urine, n = 24) but was statistically significant only for urine samples (*$P = 0.007$).

from explanted lungs (21). In the present study, LAM cells from BALF, urine, and chylous effusions reactive with anti-CD44v6 and anti-CD9 antibodies showed *TSC2* LOH. Thus, disseminated LAM cells contained prometastatic molecules that could enable their mobilization and subsequent anchorage to sites of metastasis. Phenotypic changes in metastatic cells may occur as they migrate to sites of metastasis (44). We observed that LAM cells grown from explanted lungs (as identified with the markers CD44v6/CD44), in blood (CD235a), and in BALF, urine, and chyle (CD44v6/CD9) differed in the expression of surface proteins, suggesting that LAM cells within different microenvironments have different phenotypic characteristics, which is consistent with their phenotypic heterogeneity in different tissues. In fact, human $TSC2^{-/-}$ cells are known to exhibit different morphologies in different locations. In renal AMLs, $TSC2^{-/-}$ cells appear as smooth muscle, fat, and vascular cells. LAM cells in the lungs may be spindle-shaped or epithelioid.

S-LAM is considered to be associated most frequently with mutations in *TSC2*. In our studies, *TSC2* LOH was detected in 38 of 43 (88%) patients with S-LAM, consistent with the hypothesis that more patients with S-LAM have dysfunctional *TSC2* than *TSC1*. We did not find *TSC2* LOH in five informative patients with S-LAM; two patients with S-LAM were noninformative because of their homozygosity for all five tested markers (Table 4). To increase detection of *TSC2* LOH, we did SNP-based LOH analysis on these patients using two SNPs within the *TSC2* gene. We identified LOH at the exon 40 polymorphism in BALF cell samples from one of three patients informative for two SNPs; blood and urine cell samples from this patient were not amplified well, probably due to low DNA amounts (data not shown). To determine whether or not these seven patients with S-LAM have *TSC1* abnormalities, we further assessed *TSC1* LOH but did not identify any patients with *TSC1* LOH. Failure to detect *TSC1* LOH could result from the fact that the second hit for *TSC1* may be subtle sequence changes (e.g., point mutations, small deletions), which are not detectable by LOH analysis. It is also possible that methylation may be responsible for dysregulation of the *TSC2* gene (45). The absence of *TSC1* mutations in LAM cells from patients with S-LAM suggests that pulmonary disease due to this mutation may be subclinical.

Based on earlier reports that AMLs and pulmonary LAM cells from the same patients with S-LAM have the same *TSC2* mutations and identical *TSC2* LOH patterns (13, 14), it was hypothesized that pulmonary LAM cells and AML cells could have a common genetic origin, and LAM cells could metastasize *in vivo*. Here, we described identical LOH patterns at the chromosome 16p13.3 region in LAM cells isolated from blood, urine, BALF, or chyle from the same patient in 27 of 37 (73%) cases. In 8 of 29 (23%) patients with S-LAM and 2 of 8 (25%) patients with TSC-LAM, however, LAM cells from different body fluids appeared to differ in the extent of LOH regions based on the informative microsatellites. Our data from two patients with TSC-LAM are consistent with the report that two AMLs from the same patient with TSC with multiple AMLs showed different regions of LOH on 16p13 (46). These findings from eight patients with S-LAM are discordant with a prior report (14) and suggest that in some patients with S-LAM, LAM cells may show genetic heterogeneity, which (*a*) could result from a different second mutation in cells containing the same first mutation, or (*b*) result from the introduction of new independent genetic changes in the existing LAM cells during the metastatic process, or (*c*) could represent a second novel LAM cell with two different mutations. In support of the first model, skin lesions in patients with TSC appear to arise from cells with independent second mutations in the *TSC2* genes (47). The second model of chromosomal instability appears to occur frequently in cancer cells (48). The third model is least likely. Further studies would be required to define the extent of the deletion and the identification of specific genes involved as well as the mechanism(s) of LOH (e.g., mitotic nondisjunction with reduplication of the mutant chromosome).

Among five microsatellites on chromosome 16p13.3, Kg8, closer in proximity to the *TSC2* gene than the other microsatellite markers (i.e., D16S291, D16S3395, D16S3024, and D16S521), was more frequently affected in patients with LAM (Table 3). In one case, ROH was observed at the Kg8 locus, but LOH was observed in microsatellite markers D16S3395 and D16521, which span a region telomeric to the *TSC2* locus (Table E1, *see* S-LAM1). Nearly half of patients with S-LAM showed LOH of two informative microsatellites; the patterns include LOH at Kg8 and D16S3395, two adjacent microsatellites that span the *TSC2* locus, and LOH at two distant microsatellites mapped centromerically (from D16S291 through Kg8) or telomerically (from D16S3395 through D16S521) to the *TSC2* locus or spanning the *TSC2* locus (from Kg8 through D16S521). Our data indicate that LAM cells from these patients might have loss of a larger part and a different region of chromosome 16.

An association between the presence of AMLs and lymphatic involvement and detection of *TSC2* LOH in blood and urine was also assessed, but no significant differences were found between detection of *TSC2* LOH in blood cell fractions and urine and the presence of lymphangioleiomyomas, adenopathy, or lymphangioleiomyomas/adenopathy in patients with LAM with or without AMLs (Figure E5). A statistically significant association between *TSC2* LOH in cells from urine and the presence of AMLs in patients with LAM (Figure 4) suggests that circulating LAM cells are more likely to be shed from blood into the urine in patients with LAM with AMLs than those without AMLs. Alternatively, cells from AMLs might be shed directly into urine, perhaps due to necrosis within the tumors.

Overall, we found that the presence of specific surface proteins (e.g., CD44v6, CD9, CD235a) was associated with LAM cells exhibiting *TSC2* LOH in blood, BALF, urine, and chyle, which supports, in most cases, a metastatic dissemination of LAM cells via blood and/or lymphatic circulatory systems. Contrary to previous observations, however, our data suggest that LAM cells may exhibit genetic as well as phenotypic heterogeneity in some of patients with S-LAM.

Author Disclosure: X.C. is employed by the National Institutes of Health (NIH) as a postdoctoral visiting fellow. G.P-R. does not have a financial relationship with a commercial entity that has an interest in the subject of this manuscript. Q-Y.F. does not have a financial relationship with a commercial entity that has an interest in the subject of this manuscript. M.H. is an employee of the NIH. L.S. is a full-time employee of the NIH. S.E-C. does not have a financial relationship with a commercial entity that has an interest in the subject of this manuscript. H-P.W. is an employee of the NIH. J.P.M. and J.P.M.'s spouse/life partner are employees of the NIH and owned $5,001–$10,000 in stock ownership or options in Altria within the last 12 months or at present. W.K.S. is an employee of the NIH. J-P.L. does not have a financial relationship with a commercial entity that has an interest in the subject of this manuscript. T.N.D. received more than $100,001 from the NIH in sponsored grants (R01, R21) and more than $100,001 from the Department of Defense in sponsored grants (CDMRP, DMRDP grants). J.M. has received $1,001–$5000 in patent royalties from the NIH for an invention licensed by Emiliem. J.M. is employed by the NIH.

Acknowledgment: The authors thank Dr. Martha Vaughan (NHLBI, NIH) for helpful discussions and critical review of the manuscript, and Drs. Christian Combs and Daniela Malide of the microscopy core facility for technical assistance. We thank the LAM Foundation and the Tuberous Sclerosis Alliance for their assistance in recruiting patients for our studies.

References

1. Taveira-DaSilva AM, Steagall WK, Moss J. Lymphangioleiomyomatosis. *Cancer Contr* 2006;13:276–285.
2. Urban T, Lazor R, Lacronique J, Murris M, Labrune S, Valeyre D, Cordier JF. Pulmonary lymphangioleiomyomatosis. A study of 69 patients. Groupe d'etudes et de recherche sur les maladies "Orphelines" Pulmonaires (germ"O"P). *Medicine (Baltimore)* 1999;78:321–337.
3. McCormack FX. Lymphangioleiomyomatosis: a clinical update. *Chest* 2008;133:507–516.
4. Johnson SR, Tattersfield AE. Lymphangioleiomyomatosis. *Semin Respir Crit Care Med* 2002;23:85–92.
5. Glasgow CG, Taveira-Dasilva AM, Darling TN, Moss J. Lymphatic involvement in lymphangioleiomyomatosis. *Ann N Y Acad Sci* 2008; 1131:206–214.
6. McCormack FX. Lymphangioleiomyomatosis. *MedGenMed* 2006;8:15.
7. Moss J, Avila NA, Barnes PM, Litzenberger RA, Bechtle J, Brooks PG, Hedin CJ, Hunsberger S, Kristof AS. Prevalence and clinical characteristics of lymphangioleiomyomatosis (LAM) in patients with tuberous sclerosis complex. *Am J Respir Crit Care Med* 2001; 164:669–671.
8. Costello LC, Hartman TE, Ryu JH. High frequency of pulmonary lymphangioleiomyomatosis in women with tuberous sclerosis complex. *Mayo Clin Proc* 2000;75:591–594.
9. van Slegtenhorst M, de Hoogt R, Hermans C, Nellist M, Janssen B, Verhoef S, Lindhout D, van den Ouweland A, Halley D, Young J, *et al.* Identification of the tuberous sclerosis gene *TSC1* on chromosome 9q34. *Science* 1997;277:805–808.
10. Consortium TECTS. Identification and characterization of the tuberous sclerosis gene on chromosome 16. *Cell* 1993;75:1305–1315.
11. Povey S, Burley MW, Attwood J, Benham F, Hunt D, Jeremiah SJ, Franklin D, Gillett G, Malas S, Robson EB, *et al.* Two loci for tuberous sclerosis: One on 9q34 and one on 16p13. *Ann Hum Genet* 1994;58:107–127.
12. Smolarek TA, Wessner LL, McCormack FX, Mylet JC, Menon AG, Henske EP. Evidence that lymphangiomyomatosis is caused by *TSC2* mutations: Chromosome 16p13 loss of heterozygosity in angiomyolipomas and lymph nodes from women with lymphangiomyomatosis. *Am J Hum Genet* 1998;62:810–815.
13. Carsillo T, Astrinidis A, Henske EP. Mutations in the tuberous sclerosis complex gene *TSC2* are a cause of sporadic pulmonary lymphangioleiomyomatosis. *Proc Natl Acad Sci USA* 2000;97:6085–6090.
14. Yu J, Astrinidis A, Henske EP. Chromosome 16 loss of heterozygosity in tuberous sclerosis and sporadic lymphangiomyomatosis. *Am J Respir Crit Care Med* 2001;164:1537–1540.
15. Sato T, Seyama K, Fujii H, Maruyama H, Setoguchi Y, Iwakami S, Fukuchi Y, Hino O. Mutation analysis of the *TSC1* and *TSC2* genes in Japanese patients with pulmonary lymphangioleiomyomatosis. *J Hum Genet* 2002;47:20–28.
16. Knudson AG. Hereditary cancer: two hits revisited. *J Cancer Res Clin Oncol* 1996;122:135–140.
17. Karbowniczek M, Astrinidis A, Balsara BR, Testa JR, Lium JH, Colby TV, McCormack FX, Henske EP. Recurrent lymphangiomyomatosis after transplantation: genetic analyses reveal a metastatic mechanism. *Am J Respir Crit Care Med* 2003;167:976–982.
18. Bittmann I, Rolf B, Amann G, Lohrs U. Recurrence of lymphangioleiomyomatosis after single lung transplantation: new insights into pathogenesis. *Hum Pathol* 2003;34:95–98.
19. Crooks DM, Pacheco-Rodriguez G, DeCastro RM, McCoy JP Jr, Wang JA, Kumaki F, Darling T, Moss J. Molecular and genetic analysis of disseminated neoplastic cells in lymphangioleiomyomatosis. *Proc Natl Acad Sci USA* 2004;101:17462–17467.
20. Kumasaka T, Seyama K, Mitani K, Souma S, Kashiwagi S, Hebisawa A, Sato T, Kubo H, Gomi K, Shibuya K, *et al.* Lymphangiogenesis-mediated shedding of LAM cell clusters as a mechanism for dissemination in lymphangioleiomyomatosis. *Am J Surg Pathol* 2005;29:1356–1366.
21. Pacheco-Rodriguez G, Steagall WK, Crooks DM, Stevens LA, Hashimoto H, Li S, Wang JA, Darling TN, Moss J. *TSC2* loss in lymphangioleiomyomatosis cells correlated with expression of CD44v6, a molecular determinant of metastasis. *Cancer Res* 2007;67:10573–10581.
22. Ponta H, Sherman L, Herrlich PA. CD44: From adhesion molecules to signalling regulators. *Nat Rev Mol Cell Biol* 2003;4:33–45.
23. Marhaba R, Zoller M. CD44 in cancer progression: adhesion, migration and growth regulation. *J Mol Histol* 2004;35:211–231.
24. Gunthert U, Hofmann M, Rudy W, Reber S, Zoller M, Haussmann I, Matzku S, Wenzel A, Ponta H, Herrlich P. A new variant of glycoprotein CD44 confers metastatic potential to rat carcinoma cells. *Cell* 1991;65:13–24.
25. Li S, Takeuchi F, Wang JA, Fan Q, Komurasaki T, Billings EM, Pacheco-Rodriguez G, Moss J, Darling TN. Mesenchymal-epithelial interactions involving epiregulin in tuberous sclerosis complex hamartomas. *Proc Natl Acad Sci USA* 2008;105:3539–3544.
26. Cai X, Pacheco-Rodriguez G, Fan Q, Haughey M, El-Chemaly S, Gochuico B, Samsel L, McCoy JP, Darling TN, Moss J. Identification of disseminated CD44v6^{+}/CD9^{+} cells with *TSC2* loss of heterozygosity in patients with lymphangioleiomyomatosis [abstract]. *Am J Respir Crit Care Med* 2009;179:A4339.
27. Tomlinson IP, Lambros MB, Roylance RR. Loss of heterozygosity analysis: practically and conceptually flawed? *Genes Chromosomes Cancer* 2002;34:349–353.
28. Roberts PS, Chung J, Jozwiak S, Dabora SL, Franz DN, Thiele EA, Kwiatkowski DJ. SNP identification, haplotype analysis, and parental origin of mutations in *TSC2. Hum Genet* 2002;111:96–101.
29. van Bakel I, Sepp T, Yates JR, Green AJ. An EcoRV polymorphism in exon 40 of the tuberous sclerosis 2 (TSC2) gene. *Mol Cell Probes* 1997;11:75–76.
30. Platten M, Meyer-Puttlitz B, Blumcke I, Waha A, Wolf HK, Nothen MM, Louis DN, Sampson JR, von Deimling A. A novel splice site associated polymorphism in the tuberous sclerosis 2 (TSC2) gene may predispose to the development of sporadic gangliogliomas. *J Neuropathol Exp Neurol* 1997;56:806–810.
31. Henske EP. Metastasis of benign tumor cells in tuberous sclerosis complex. *Genes Chromosomes Cancer* 2003;38:376–381.
32. Pantel K, Brakenhoff RH, Brandt B. Detection, clinical relevance and specific biological properties of disseminating tumour cells. *Nat Rev Cancer* 2008;8:329–340.
33. Pantel K, Alix-Panabieres C, Riethdorf S. Cancer micrometastases. *Nat Rev Clin Oncol* 2009;6:339–351.
34. Mostert B, Sleijfer S, Foekens JA, Gratama JW. Circulating tumor cells (CTCs): detection methods and their clinical relevance in breast cancer. *Cancer Treat Rev* 2009;35:463–474.
35. Zoller M. Tetraspanins: push and pull in suppressing and promoting metastasis. *Nat Rev Cancer* 2009;9:40–55.
36. Boucheix C, Duc GH, Jasmin C, Rubinstein E. Tetraspanins and malignancy. *Expert Rev Mol Med* 2001;2001:1–17.
37. Wright MD, Moseley GW, van Spriel AB. Tetraspanin microdomains in immune cell signalling and malignant disease. *Tissue Antigens* 2004; 64:533–542.
38. Lazo PA. Functional implications of tetraspanin proteins in cancer biology. *Cancer Sci* 2007;98:1666–1677.
39. Maecker HT, Todd SC, Levy S. The tetraspanin superfamily: molecular facilitators. *FASEB J* 1997;11:428–442.
40. Stipp CS, Kolesnikova TV, Hemler ME. Functional domains in tetraspanin proteins. *Trends Biochem Sci* 2003;28:106–112.
41. Hemler ME. Tetraspanin proteins mediate cellular penetration, invasion, and fusion events and define a novel type of membrane microdomain. *Annu Rev Cell Dev Biol* 2003;19:397–422.
42. Hemler ME. Tetraspanin functions and associated microdomains. *Nat Rev Mol Cell Biol* 2005;6:801–811.
43. Levy S, Shoham T. Protein-protein interactions in the tetraspanin web. *Physiology (Bethesda)* 2005;20:218–224.

44. Gupta GP, Massague J. Cancer metastasis: building a framework. *Cell* 2006;127:679–695.
45. Lesma E, Sirchia SM, Ancona S, Carelli S, Bosari S, Ghelma F, Montanari E, Di Giulio AM, Gorio A. The methylation of the TSC2 promoter underlies the abnormal growth of TSC2 angiomyolipoma-derived smooth muscle cells. *Am J Pathol* 2009;174:2150–2159.
46. Henske EP, Neumann HP, Scheithauer BW, Herbst EW, Short MP, Kwiatkowski DJ. Loss of heterozygosity in the tuberous sclerosis (*TSC2*) region of chromosome band 16p13 occurs in sporadic as well as TSC-associated renal angiomyolipomas. *Genes Chromosomes Cancer* 1995;13:295–298.
47. Wang J, Li S, Fan Q, Pacheco-Rodriguez G, Moss J, Darling TN. Independent origins for multifocal skin tumors in tuberous sclerosis complex [abstract]. *J Invest Dermatol* 2007;127:S23.
48. Loeb LA, Bielas JH, Beckman RA. Cancers exhibit a mutator phenotype: Clinical implications. *Cancer Res* 2008;68:3551–3557.