CHEST

Original Research

DIFFUSE LUNG DISEASE

Sirolimus Decreases Circulating Lymphangioleiomyomatosis Cells in Patients With Lymphangioleiomyomatosis

Xiong Cai, PhD; Gustavo Pacheco-Rodriguez, PhD; Mary Haughey, RN, BSN; Leigh Samsel, MS; Suowen Xu, PhD; Hai-Ping Wu, BS; J. Philip McCoy, PhD; Mario Stylianou, PhD; Thomas N. Darling, MD, PhD; and Joel Moss, MD, PhD, FCCP

Background: **Lymphangioleiomyomatosis (LAM), sporadic or in women with tuberous sclerosis complex (TSC), is characterized by cystic lung destruction, lymphatic involvement (eg, chylous pleural effusions, lymphangioleiomyomas), and renal angiomyolipomas (AMLs). The multisystem manifestations of LAM appear to result from metastatic dissemination of LAM cells bearing inactivating mutations or having loss of heterozygosity (LOH) of the tumor suppressor genes *TSC1* or *TSC2*, which leads to hyperactivation of the mammalian target of rapamycin. Sirolimus slows the decline of lung function, reduces chylous effusions, and shrinks the size of AMLs. The purpose of this study was to determine the effect of sirolimus on circulating LAM cells.**

Methods: **Cells from blood were isolated by a density-gradient fractionation system and from urine and chylous effusions by centrifugation. Blood cells were incubated with anti-CD45-fluorescein isothiocyanate (FITC) and anti-CD235a-R-phycoerythrin (PE) antibodies, and urine and chylous effusion cells were incubated with anti-CD44v6-FITC and anti-CD9-R-PE antibodies. Cells were sorted and analyzed for *TSC2* LOH.**

Results: **LAM cells with *TSC2* LOH were identified in 100% of blood specimens and 75% of urine samples from patients before therapy. Over a mean duration of 2.2 ± 0.4 years of sirolimus therapy, detection rates of LAM cells were significantly decreased to 25% in blood ($P < .001$) and 8% in urine ($P = .003$). Following therapy, a greater loss of circulating LAM cells was seen in postmenopausal patients ($P = .025$).**

Conclusions: **Patients receiving sirolimus had a progressive loss of circulating LAM cells that depended on time of treatment and menopausal status.** ***CHEST 2014; 145(1):108–112***

Abbreviations: AML = angiomyolipoma; LAM = lymphangioleiomyomatosis; LOH = loss of heterozygosity; mTOR = mammalian target of rapamycin; NIH = National Institutes of Health; PCR = polymerase chain reaction; TSC = tuberous sclerosis complex

Lymphangioleiomyomatosis (LAM), a rare multisystem disorder affecting primarily women of child-bearing age, is characterized by cystic lung destruction, lymphatic involvement (eg, chylous pleural effusions, lymphangioleiomyomas), and renal angiomyolipomas (AMLs).[1] LAM occurs sporadically or in association with tuberous sclerosis complex (TSC), an autosomal dominant disorder. The multisystem manifestations of LAM are believed to result from metastatic dissemination of abnormal smooth muscle-like LAM cells bearing inactivating mutations or having loss of heterozygosity (LOH) of one of the two tumor suppressor genes *TSC1* and *TSC2*,[2] which leads to hyperactivation of mammalian target of rapamcyin (mTOR).[3,4] mTOR controls cell proliferation, growth, and motility.[5] Because mutations in genes encoding proteins upstream of mTOR and regulatory abnormalities in critical cellular pathways lead to hyperactivation of mTOR, rapamycin (sirolimus) and its analogs are being used to treat cancers (eg, renal cell carcinoma), hamartomas (eg, AMLs), vascular diseases (eg, antirestenosis following angioplasty), and allograft rejection (eg, immunosuppression).[6-8] In addition, rapamycin has been used to target metastatic processes in animals models.[9,10]

Patients treated with sirolimus have shown improvement in lung function,[8] reduction of chylous effusions,[11] and decrease in the size of AMLs.[7] Although

sirolimus blocks proliferation by arresting the cell cycle,[12] effects of sirolimus on circulating cells, which may be critical to metastasis and tumor dissemination, have not been demonstrated in human disease. Here, we studied the effect of sirolimus on circulating LAM cells found in patients with LAM.[13,14] To determine the effect of sirolimus on circulating LAM cells, we investigated the presence of LAM cells in the blood, chylous effusions, and urine in patients receiving and not receiving sirolimus therapy.

Materials and Methods

Study Design

Twenty-three patients with LAM were enrolled between 2007 and 2012 at the National Institutes of Health (NIH) 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, radiographic, and histopathologic findings. Patients with only high-resolution CT scan-compatible cystic disease were not judged to have LAM, unless a biopsy specimen was obtained or the patients had consistent clinical findings (eg, TSC, extrapulmonary manifestations [AMLs, lymphangioleiomyomas, chylous pleural effusions, or ascites]). Local physicians prescribed sirolimus and adjusted the dose to maintain serum levels between 5 and 15 ng/mL. Samples of blood, urine, and chylous effusions were collected from patients before and after initiation of sirolimus.

Enrichment and Isolation of Circulating LAM Cells by Fluorescence-Activated Cell Sorting

Blood (50-75 mL) was fractionated by density-gradient centrifugation on OncoQuick columns (Greiner Bio-One) as previously described.[14] Urine (500 mL) from a 24-h collection and chylous effusions (100 mL) were centrifuged (300*g*, 10 min, 4°C). Enriched cells from blood were incubated for 30 min at room temperature with anti-CD45-fluorescein isothiocyanate (clone HI30) and anti-CD235a-R-phycoerythrin (clone GA-R2) antibodies (BD Biosciences). Cells from urine and chylous effusions were reacted with anti-CD44v6-fluorescein isothiocyanate (clone VFF-7) (Invitrogen, Life Technologies Corporation) and anti-CD9-R-phycoerythrin (M-L13) antibodies (BD Biosciences). After incubation, cells were washed once in phosphate-buffered saline and analyzed and sorted as previously described.[14]

Polymerase Chain Reaction Analysis of LOH

Genomic DNA was isolated from whole blood and unsorted or sorted cells with the use of the QIAamp DNA Micro Kit (QIAGEN) and amplified at loci D16S291, Kg8, D16S3395, D16S3024, and D16S521 on chromosome 16p13.3 as previously described.[13,14] Polymerase chain reaction (PCR) was performed in 10-μL mixtures containing 1 × PCR Gold Buffer, 1.5 mmol/L $MgCl_2$, 250 μmol/L each deoxynucleoside triphosphate, 0.8 μmol/L primers, and 2 units AmpliTaq Gold DNA polymerase (Applied Biosystems, Life Technologies Corporation) with a Veriti 96-Well Thermal Cycler (Applied Biosystems) with initial denaturation at 95°C for 5 min followed by 35 cycles of 95°C (30 s), 55°C (45 s), and 72°C (45 s) and a final extension at 72°C for 2 min. PCR products were analyzed on a 3100 Genetic Analyzer (Applied Biosystems). Q^{LOH} was determined by comparing the ratio of fluorescence intensities of each allele in putative LAM (L) cells to that in whole blood (N) from the same patient with (L1/L2)/(N1/N2), where L1 or N1 is the minor allele and L2 or N2 the other. Q^{LOH} values < 0.5 or > 0.62 were scored as LOH or retention of heterozygosity, respectively, whereas no definite decision was made with Q^{LOH} values 0.5 to 0.62.

Statistical Analysis

We used generalized linear models with repeated measures to compare the binary outcome variable of detection of *TSC2* LOH before and after sirolimus therapy and between blood and urine samples. A first-order autoregressive structure was used to model the correlations in the repeated measurements. We performed a multivariate analysis where the combined data of both fluid tests were used along with treatment time and menopausal status to determine factors associated with detection of LAM cells. ORs and 95% CIs were derived. However, the comparison of detection of *TSC2* LOH before and after sirolimus therapy could not be performed with blood samples because the detection rate of LOH before sirolimus therapy in these patients was 100%, so no estimates could be derived. We compared differences in detection rates of *TSC2* LOH before and after sirolimus therapy with Fisher exact test. Continuous data are reported as mean ± SEM. Two-tailed statistical tests were used, and $P < .05$ was considered significant. All statistical analyses were performed with the SPSS version 15.0 for Windows (IBM Corporation) software.

Results

Twenty-three patients with LAM who fit the study inclusion criteria were enrolled between 2007 and 2012 at the NIH Clinical Center. Baseline demographic and clinical characteristics are shown in Table 1. Samples of blood, urine, and chylous effusions were collected at NIH before and during sirolimus therapy. Sirolimus was prescribed by local physicians and the dose adjusted to maintain serum levels between 5 and 15 ng/mL. Cells from blood, chylous effusions, and urine were sorted on the basis of cell surface markers (CD235a, CD45, CD44v6, CD9) that have been

Manuscript received May 3, 2013; revision accepted August 1, 2013.
Affiliations: From the Cardiovascular and Pulmonary Branch (Drs Cai, Pacheco-Rodriguez, Xu, and Moss and Mss Haughey and Wu), Flow Cytometry Core Facility (Ms Samsel and Dr McCoy), and Office of Biostatistics Research, Division of Cardiovascular Science (Dr Stylianou), National Heart, Lung, and Blood Institute, National Institutes of Health; and the Department of Dermatology (Dr Darling), Uniformed Services University of the Health Sciences, Bethesda, MD.
Dr Cai is currently at the School of Pharmaceutical Sciences, Nanjing University of Chinese Medicine (Nanjing, Jiangsu, China).
Funding/Support: This study was funded in part by the Intramural Research Program of the National Institutes of Health, National Heart, Lung, and Blood Institute [to Dr Moss], and R01-AR062080 [to Dr Darling].
Correspondence to: Joel Moss, MD, PhD, FCCP, Cardiovascular and Pulmonary Branch, National Heart, Lung, and Blood Institute, National Institutes of Health, Bldg 10, Room 6D05, MSC-1590, 9000 Rockville Pike, Bethesda, MD 20892-1590; e-mail: mossj@nhlbi.nih.gov

DOI: 10.1378/chest.13-1071

Table 1—*Baseline Demographic and Clinical Characteristics of Patients With LAM*

Characteristic	Value
Age, mean ± SEM, y	
At presentation with LAM-related symptoms	36.8 ± 8.6
At diagnosis of LAM symptoms	40.9 ± 8.4
At enrollment in the NHLBI LAM protocol	42.7 ± 8.1
At initiation of sirolimus therapy	47.2 ± 10.0
At most recent follow-up visit	49.7 ± 9.3
Interval from diagnosis to initiation of sirolimus therapy, mean ± SD, y	6.3 ± 6.7
Race	
White	20 (87)
African American	1 (4)
Asian	1 (4)
Unknown	1 (4)
Clinical features	
Tuberous sclerosis complex	4 (17)
Postmenopausal	9 (39)
History of pneumothorax	7 (30)
History of dyspnea	15 (65)
Requirement for oxygen therapy	9 (39)
Chylous pleural effusions	10 (43)
Ascites	6 (26)
Lymphangioleiomyomas	16 (70)
Lymphadenopathy	6 (26)
Renal angiomyolipoma	7 (30)

Data are presented as No. (%) unless otherwise indicated. LAM = lymphangioleiomyomatosis; NHLBI = National Heart, Lung, and Blood Institute.

shown previously to identify the LAM cells in body fluids and cultured lung cells[13-15] and has enabled isolation of circulating LAM cells characterized by *TSC2* LOH.[13,14] Because cells with *TSC1* or *TSC2* mutations appear to be phenotypically different depending on location, LAM cells are more accurately defined as cells possessing a genetic alteration within the *TSC2* or *TSC1* locus. We isolated $CD235a^{+}CD45^{-}$ and $CD235^{-}CD45^{-}$ cells from blood and $CD44v6^{+}CD9^{+}$ and $CD44v6^{-}/C9^{-}$ cells from urine and chyle.[13,14] To determine the presence of LAM cells, we isolated DNA from cell populations and tested five microsatellite repeats related to the *TSC2* region on chromosome 16 (D16S521, D16S3024, D16S3395, Kg8, and D16S291)[13,14] by PCR and compared the levels of each allele PCR product from each repeat in isolated cell populations with DNA extracted from whole blood. Sirolimus did not appreciably affect the levels of LAM cell surface proteins used in the isolation, and sirolimus did not block the ability to detect LAM cells in the OncoQuick fractionation (e-Appendix 1). A LAM cell population was identified as the one with LOH (LOH scored as $Q^{LOH} \leq 0.5$).

Twelve patients with LAM were identified before they started sirolimus therapy. It was determined that all 12 had circulating LAM cells with *TSC2* LOH in blood. We also identified LAM cells in the urine of nine of the 12 patients (75%). After a mean duration of 2.2 ± 0.4 years of sirolimus therapy, LAM cells were detected in the blood of only three of 12 patients (25%, $P < .001$) and in urine specimens of one patient (8%) ($P = .003$) (Fig 1A). We were unable to detect LAM cells in chylous effusions from two patients after 1 year of treatment (data not shown), which is consistent with the decrease of chylous effusions observed previously.[11] These data are consistent with the hypothesis that sirolimus decreased the number of disseminated LAM cells.

We also analyzed samples from patients with LAM who had initiated sirolimus therapy prior to being enrolled in the study. In these 11 patients who had been taking sirolimus for about 1.0 ± 0.3 years, we observed LAM cells in eight blood samples (73%) and in four urine specimens (36%). We followed these

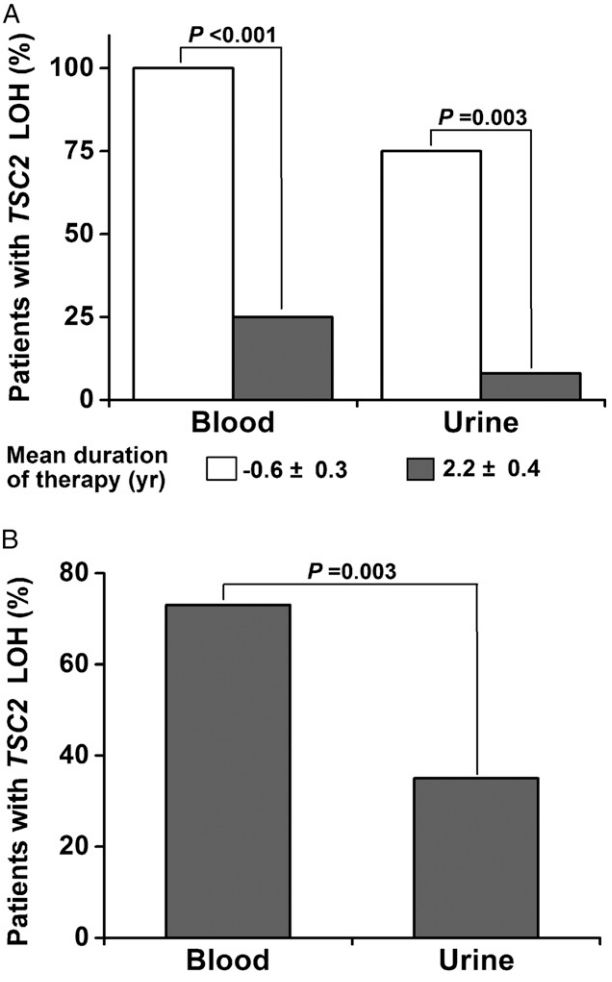

FIGURE 1. Time-dependent effect of sirolimus therapy on detection of circulating cells with *TSC2* LOH in blood and urine specimens from patients with lymphangioleiomyomatosis (LAM). A, Significant decrease in detection rate of LAM cells with *TSC2* LOH in blood and urine from 12 patients receiving sirolimus therapy with a mean follow-up of −0.6 ± 0.3 to 2.2 ± 0.4 y. B, Detection of LAM cells with *TSC2* LOH decreased more rapidly in urine samples than in blood samples within 1 y of sirolimus therapy. LOH = loss of heterozygosity.

patients for about 3 years of therapy (mean duration, 2.9 ± 0.6 years) and found that LAM cells were detected in only two blood samples (18%) ($P = .030$) and in none of the urine samples ($P = .090$). Therefore, the ability to detect LAM cells in blood was lower after 3 years of treatment with sirolimus than after 1 year, and this trend was also apparent in urine.

In all patients, including those seen prior to initial therapy, the percentage of patients with circulating LAM cells diminished within 1 year after beginning sirolimus. Sixteen of 22 patients (73%) showed LOH in blood, and seven of 20 patients (35%) showed LOH in urine; one patient had no data for blood, and three had no data for urine within 1 year ($P = .003$) (Fig 1B). The ORs of not detecting LAM cells in urine and blood samples 1 year after initiation of sirolimus therapy was 3.41 ($P = .001$) and 2.23 ($P = .001$), respectively. In response to sirolimus therapy, the ability to detect LAM cells was lost more rapidly from urine than from blood. In patients treated with sirolimus for a mean duration of 1.6 ± 0.8 years, five did not show the presence of LAM cells; after 2.8 ± 0.4 years of sirolimus therapy, cells were not detected in 18. Thus, longer duration of therapy resulted in fewer patients with detectable circulating LAM cells.

To further explore the relationship among LAM cells, treatment duration, and other factors such as menopausal status, we used generalized linear models with repeated measurements to compare the binary outcome variable of detection of LOH before and after sirolimus therapy and between blood and urine samples. A first-order autoregressive structure was used to model the correlations in the repeated measurements. However, the comparison of pretreatment and posttreatment could not be performed in blood samples because the pretreatment LAM cell (*TSC2* LOH) detection rate in this cohort was 100%, so no estimates could be derived. The OR for not detecting LAM cells in urine samples 1 year after initiation of sirolimus therapy was 3.41 ($P = .001$). We have continued with the determination of LAM cells in patients receiving sirolimus treatment, and they continue to show a lack of LAM cells in blood and urine in a time-dependent manner.

We analyzed the difference in detection of circulating cells with *TSC2* LOH between premenopausal and postmenopausal patients with LAM. In the untreated group of 50 patients,[14] the detection rate of *TSC2* LOH was similar in blood (91% vs 94%) and urine (69% vs 78%) between 32 premenopausal and 18 postmenopausal patients. The current study included 14 premenopausal and nine postmenopausal patients treated with sirolimus. A multivariate model revealed that the number of LAM cells was decreased with sirolimus therapy ($P = .038$), and the decrease in LOH detection was correlated with duration of therapeutic intervention ($P < .001$); the loss of circulating LAM cells was most marked in postmenopausal patients ($P = .025$). These data suggest that sirolimus may reduce circulating LAM cells in a time- and hormone-dependent manner.

Discussion

We studied the effect of sirolimus on phenotypically distinct circulating LAM cells. Patients treated with sirolimus had a decrease in circulating LAM cells in blood, chylous effusions, and urine that depended on time of treatment and the hormonal status of the patient. The LAM cells were identified by LOH in the tumor suppressor gene *TSC2*. This assay was used because nucleotide polymorphisms flanking the tumor suppressor gene, which were the basis for the LOH determinations, were shown previously to be associated with mutations in the gene.[2,16] We have shown in prior studies that circulating LAM cells from different fluids possess distinct phenotypes based on antibody reactivity,[14] and we now report that sirolimus affects all the different types of circulating LAM cells.

Animal models for LAM support a role for estrogen.[17] In fact, it has been postulated that LAM cell dissemination and survival in the circulation could be promoted by estrogen.[17] On the other hand, patients with LAM are treated with sirolimus because of the hyperactivation of mTOR as a consequence of the genetic alteration of *TSC2* or *TSC1*[3]; however, a relationship between estrogen and mTOR activation has not been defined. It is possible that the menopausal status of the patient could affect the efficacy of sirolimus. In the present study, we show that following initiation of sirolimus therapy, postmenopausal patients are more likely to show a loss of circulating LAM cells.

In conclusion, the data support the hypothesis that sirolimus reduces circulating LAM cells and thereby may inhibit metastasis and block disease progression. It has been shown that circulating tumor cells from patients with breast, prostate, and other cancers are reduced in response to therapy.[18] The data also show that duration of sirolimus therapy in patients with LAM is associated with a decreased ability to detect circulating LAM cells in blood, urine, and chylous effusions. LAM cells appear to be cleared more rapidly from urine than from blood. The data on premenopausal and postmenopausal women treated with sirolimus further support a role for estrogen in maintaining circulating LAM cells. The data suggest that an estrogen inhibitor (eg, blocking estrogen synthesis, antiestrogen) may be beneficial in association with an mTOR inhibitor in blocking the potential for metastasis and tumor progression.

Acknowledgments

Author contributions: Drs Cai, Pacheco-Rodriguez, Stylianou, and Moss had full access to all of the data in the study and take responsibility for the integrity of the data and the accuracy of the data analysis.
Dr Cai: contributed to carrying out the research, analyzing the data, and writing the manuscript.
Dr Pacheco-Rodriguez: contributed to the research design, sample preparation, and writing of the manuscript.
Ms Haughey: contributed to the patient recruitment, data analysis, and review of the manuscript.
Ms Samsel: contributed to the flow cytometric analysis, cell sorting, and review of the manuscript.
Dr Xu: contributed to the sample preparation and review of the manuscript.
Ms Wu: contributed to the sample preparation and review of the manuscript.
Dr McCoy: contributed to the flow cytometric analysis, cell sorting, and review of the manuscript.
Dr Stylianou: contributed to the statistical analyses and review of the manuscript.
Dr Darling: contributed to the research design and review of the manuscript.
Dr Moss: contributed to the research design, discussion of experiments, data analysis, and writing of the manuscript.
Financial/nonfinancial disclosures: The authors have reported to *CHEST* that no potential conflicts of interest exist with any companies/organizations whose products or services may be discussed in this article.
Role of sponsors: The sponsor had no role in the design of the study, the collection and analysis of the data, or the preparation of the manuscript.
Other contributions: We thank Martha Vaughan, MD (National Heart, Lung, and Blood Institute, NIH), for helpful discussions and critical review of the manuscript. We also thank The LAM Foundation and the Tuberous Sclerosis Alliance for assistance in recruiting patients for the studies.
Additional information: The e-Appendix can be found in the "Supplemental Materials" area of the online article.

References

1. Ryu JH, Moss J, Beck GJ, et al; NHLBI LAM Registry Group. The NHLBI lymphangioleiomyomatosis registry: characteristics of 230 patients at enrollment. *Am J Respir Crit Care Med*. 2006;173(1):105-111.
2. 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 U S A*. 2000;97(11):6085-6090.
3. Goncharova EA, Goncharov DA, Eszterhas A, et al. Tuberin regulates p70 S6 kinase activation and ribosomal protein S6 phosphorylation. A role for the TSC2 tumor suppressor gene in pulmonary lymphangioleiomyomatosis (LAM). *J Biol Chem*. 2002;277(34):30958-30967.
4. Huang J, Manning BD. The TSC1-TSC2 complex: a molecular switchboard controlling cell growth. *Biochem J*. 2008; 412(2):179-190.
5. Laplante M, Sabatini DM. mTOR signaling in growth control and disease. *Cell*. 2012;149(2):274-293.
6. Benjamin D, Colombi M, Moroni C, Hall MN. Rapamycin passes the torch: a new generation of mTOR inhibitors. *Nat Rev Drug Discov*. 2011;10(11):868-880.
7. Bissler JJ, McCormack FX, Young LR, et al. Sirolimus for angiomyolipoma in tuberous sclerosis complex or lymphangioleiomyomatosis. *N Engl J Med*. 2008;358(2):140-151.
8. McCormack FX, Inoue Y, Moss J, et al; National Institutes of Health Rare Lung Diseases Consortium; MILES Trial Group. Efficacy and safety of sirolimus in lymphangioleiomyomatosis. *N Engl J Med*. 2011;364(17):1595-1606.
9. Guba M, von Breitenbuch P, Steinbauer M, et al. Rapamycin inhibits primary and metastatic tumor growth by antiangiogenesis: involvement of vascular endothelial growth factor. *Nat Med*. 2002;8(2):128-135.
10. Zhou H, Huang S. Role of mTOR signaling in tumor cell motility, invasion and metastasis. *Curr Protein Pept Sci*. 2011;12(1):30-42.
11. Taveira-DaSilva AM, Hathaway O, Stylianou M, Moss J. Changes in lung function and chylous effusions in patients with lymphangioleiomyomatosis treated with sirolimus. *Ann Intern Med*. 2011;154(12):797-805.
12. Schmelzle T, Hall MN. TOR, a central controller of cell growth. *Cell*. 2000;103(2):253-262.
13. Crooks DM, Pacheco-Rodriguez G, DeCastro RM, et al. Molecular and genetic analysis of disseminated neoplastic cells in lymphangioleiomyomatosis. *Proc Natl Acad Sci U S A*. 2004; 101(50):17462-17467.
14. Cai X, Pacheco-Rodriguez G, Fan QY, et al. Phenotypic characterization of disseminated cells with TSC2 loss of heterozygosity in patients with lymphangioleiomyomatosis. *Am J Respir Crit Care Med*. 2010;182(11):1410-1418.
15. Pacheco-Rodriguez G, Steagall WK, Crooks DM, et al. TSC2 loss in lymphangioleiomyomatosis cells correlated with expression of CD44v6, a molecular determinant of metastasis. *Cancer Res*. 2007;67(21):10573-10581.
16. European Chromosome 16 Tuberous Sclerosis Consortium. Identification and characterization of the tuberous sclerosis gene on chromosome 16. *Cell*. 1993;75(7):1305-1315.
17. Yu JJ, Robb VA, Morrison TA, et al. Estrogen promotes the survival and pulmonary metastasis of tuberin-null cells. *Proc Natl Acad Sci U S A*. 2009;106(8):2635-2640.
18. Cristofanilli M, Budd GT, Ellis MJ, et al. Circulating tumor cells, disease progression, and survival in metastatic breast cancer. *N Engl J Med*. 2004;351(8):781-791.