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Genetic Heterogeneity of Circulating Cells from Patients with Lymphangioleiomyomatosis with and without Lung Transplantation

To the Editor:

Lymphangioleiomyomatosis (LAM) is a multisystem disease characterized by lung destruction, lymphatic abnormalities, and abdominal tumors (e.g., angiomyolipomas [AMLs]) (1). LAM may present sporadically or in association with tuberous sclerosis complex (TSC) and is found primarily with genetic alterations in the tumor suppressor gene *TSC2* (1). *TSC2* loss of heterozygosity (LOH) was first shown in AMLs and retroperitoneal lymph nodes isolated from patients with sporadic LAM (2). LAM cells are believed to be clonal, as the same pattern of *TSC2* LOH is seen in different organs (3, 4). LAM cells can metastasize (5, 6), and circulating LAM cells have been isolated from blood, urine, bronchoalveolar lavage fluid, and chylous effusions (7, 8). We reported 10 cases in which LAM cells from different body fluids showed differences in LOH patterns (8), suggesting that a patient may harbor different LAM cell clones. Here, we find that circulating LAM cells may exhibit extensive genetic heterogeneity.

Blood and urine samples were collected from 65 patients with LAM, 44 of whom have been studied previously (8, 9). The methods for fluorescence-activated cell sorting of circulating cells from blood and urine and for LOH determination are in the online supplement. LAM cells are defined genetically by *TSC2* LOH and phenotypically by reactivity to specific cell-surface markers. CD45 and CD235a help to identify LAM cells in blood (7, 8), whereas CD44v6 and CD9 are used with urine (8). The pattern of LOH of five microsatellite markers spanning the *TSC2* locus was noted.

On examination of *TSC2* LOH patterns seen in circulating cells from blood from 45 patients with LAM analyzed at one visit, 31 patients showed LOH in both blood subpopulations (Tables 1 and 2). Eight had a different pattern of LOH in the $CD45^-$, $CD235a^-$ population than in the $CD45^-$, $CD235a^+$ population (*see* Figure E1 in the online supplement; L80, L110, L363, L554, L47, L324, L697, L56). Age and TSC status did not affect the detection of genetic heterogeneity (Tables 1 and 2). Therefore, 25.8% of patients who showed LOH in both populations isolated from blood had evidence of circulating cells with different LOH patterns, suggesting that a single patient may have different clones of LAM cells.

LOH patterns were compared in urine and blood in 45 patients seen for one visit (Table 3). Ten patients (33.3% of patients who showed LOH in urine) showed differences in LOH

Table 1. Allelic Patterns Seen in Cells Isolated from Blood (Populations $CD45^-$, $CD235a^-$, and $CD45^-$, $CD235a^+$) of Patients with One Visit: Overall Distribution of *TSC2* Loss of Heterozygosity/Retention of Heterozygosity

TSC2 Status	No. (%) of Patients*	Mean Age ± SD (*yr*)	TSC (*n*)
ROH	4 (8.9)	50.1 ± 4.5	0
LOH in $CD45^-$, $CD235a^-$	2 (4.4)	45.3 ± 9.3	0
LOH in $CD45^-$, $CD235a^+$	8 (17.8)	52.1 ± 14.4	3
LOH in $CD45^-$, $CD235a^-$ and $CD45^-$, $CD235a^+$	31 (68.9)	47.5 ± 11.0	6

Definition of abbreviations: LOH = loss of heterozygosity; ROH = retention of heterozygosity; TSC = tuberous sclerosis complex.
TSC column shows the number of patients in that category who have tuberous sclerosis.
*N = 45 patients.

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

Table 2. Allelic Patterns Seen in Cells Isolated from Blood (Populations $CD45^-$, $CD235a^-$, and $CD45^-$, $CD235a^+$) of Patients with One Visit: Distribution of Allelic Patterns in Those with Loss of Heterozygosity in Both $CD45^-$, $CD235a^-$ and $CD45^-$, $CD235a^+$ Populations

Allelic Pattern	No. (%) of Patients	Mean Age ± SD (*yr*)	TSC (*n*)
$CD45^-$, $CD235a^-$ = $CD45^-$, $CD235a^+$	23 (74.2)	47.4 ± 11.8	4
$CD45^-$, $CD235a^-$ ≠ $CD45^-$, $CD235a^+$	8 (25.8)	47.7 ± 8.9	2

Definition of abbreviations: LOH = loss of heterozygosity; ROH = retention of heterozygosity; TSC = tuberous sclerosis complex.
TSC column shows the number of patients in that category who have tuberous sclerosis. Allelic pattern refers to the combination of ROH and LOH seen across the five markers tested (D16S521, D16S3024, D16S3395, Kg8, and D16S291). $CD45^-$, $CD235a^-$ = $CD45^-$, $CD235a^+$: the pattern is the same in cells from populations $CD45^-$, $CD235a^-$ and $CD45^-$, $CD235a^+$ (*see* L139 or L186 in Figure E1 for example); $CD45^-$, $CD235a^-$ ≠ $CD45^-$, $CD235a^+$: the pattern is different in cells from population $CD45^-$, $CD235a^-$ versus $CD45^-$, $CD235a^+$ (*see* L47 or L80 in Figure E1 for example).

pattern between blood subpopulations and urine (Figure E1; L80, L110, L544, L158, L554, L403, L417, L484, L324, and L363). This genetic heterogeneity was independent of age and TSC status (Table 3).

Twenty patients were monitored for several visits (Figures E1B and E1C). These patients also showed different patterns of allelic loss in blood subpopulations or blood versus urine over time. Thus, there may be a wide range of heterogeneity in LAM cells, both phenotypically (i.e., expression of proteins on the cell surface) and genetically (i.e., patterns of *TSC2* allelic loss).

Table 3. Differences in Allelic Patterns of Cells Isolated from Blood (CD45, CD235a) versus Urine (CD44v6, CD9) of Patients with One Visit

TSC2 Status	No. (%) of Patients*	Mean Age ± SD (*yr*)	TSC (*n*)
Allelic patterns in blood are same as those in urine	20 (44.4)	46.9 ± 12.2	3
Allelic patterns in blood are different from those in urine†	7 (15.6)	52.0 ± 11.4	3
Blood has extra allelic pattern‡	3 (6.7)	54.2 ± 2.6	0
Urine is ROH or NA	15 (33.3)	47.6 ± 10.3	3

Definition of abbreviations: NA = not amplified; ROH = retention of heterozygosity; TSC = tuberous sclerosis complex.
TSC column shows the number of patients in that category who have tuberous sclerosis.
*N = 45 patients.
†Blood and urine have completely different allelic patterns. For examples, see L324 or L363 in Figure E1.
‡One of the populations of blood ($CD45^-$, $CD235a^-$ or $CD45^-$, $CD235a^+$) had the same allelic pattern as urine, whereas the other population of blood had a different pattern. For examples, see L80, L110, or L554 in Figure E1.

We also isolated circulating LAM cells from patients after bilateral lung transplantation. Blood and urine were collected from four patients post-transplant. No circulating cells were isolated from the blood or urine of L63, whereas L56 and L697 had circulating LAM cells in blood but not in urine, and L487 had LAM cells in both (Figure E1). The patients with circulating LAM cells also showed heterogeneity in allelic patterns. These data show for the first time that patients with LAM after bilateral lung transplantation have circulating LAM cells and that these cells are genetically different. Although recipient LAM cells have been found in the donor lung after single lung transplantation (6), the origin of the LAM cell is not known. LAM cells have been postulated to arise from AMLs, the uterus, or the axial lymphatics (1). It is also possible that the lung was the primary tumor source and that the circulating cells detected in these patients receiving bilateral transplant are from micrometastases that were dormant (10).

Cell cultures from explanted lungs from three different patients were used as a source of LAM cells (11–13). We have had success isolating *TSC2* LOH cells from these mixtures based on chemokine-stimulated mobility (12, 13) and reactivity to anti-CD44v6 antibodies and a variety of anti–cell-surface protein antibodies (listed in the online supplement) (7, 12, 13). Cell populations of cultures B2305R and B1705R showed retention of allele one of the D16S3395 marker 90% of the time, whereas allele two was retained 10% of the time (Figure E2). The third LAM cell mixture (BBI9054R) also showed genetic heterogeneity. These data indicate that the heterogeneous cell mixtures are heterogeneous not only because cells with wild-type *TSC2* are present but also because they contain cells with different patterns of *TSC2* LOH. These cells may reflect genetic instability of cell culture or they may be representative of the LAM cells present in the explanted lungs.

The results of our examination of circulating and cultured LAM cells and the characterization of their genetic heterogeneity differ from previous studies of solid tissues (3–5). In this study, we looked at LAM cells from blood and found that 25.8% of those with LAM cells in both populations had different LOH patterns (Table 2). Allelic patterns also differed between blood and urine and in the same body fluid over time, such that 26 of 65 (40.0%) patients analyzed showed heterogeneity in allelic patterns of isolated cells. These data suggest that multiple clones of LAM cells may exist in different body fluids and over time.

Many human cancers have great intratumor heterogeneity in morphology, cell surface marker expression, and metastatic potential (14). Clonal heterogeneity has been shown in breast, colon, bladder, and prostate carcinomas (reviewed in Reference 14). Our study suggests that the genetic heterogeneity seen in circulating LAM cells, whether due to multiclonal origin or genetic instability over time, is consistent with a more recent model of LAM, wherein the disease is defined as a low-grade neoplasm (15). ■

Author disclosures are available with the text of this letter at www.atsjournals.org.

Wendy K. Steagall, Ph.D.
Li Zhang, Ph.D.
Xiong Cai, Ph.D.*

Gustavo Pacheco-Rodriguez, Ph.D.
Joel Moss, M.D., Ph.D.
National Institutes of Health
Bethesda, Maryland

*Current address: Institute of TCM Diagnostics, Hunan University of Chinese Medicine, 113 Shaoshan Avenue, Yuhua District, Changsha, Hunan 410007, China.

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Attenuation of Obstructive Sleep Apnea and Overnight Rostral Fluid Shift by Physical Activity

To the Editor:

Physical activity attenuates obstructive sleep apnea (OSA) severity, as assessed by the frequency of apneas and hypopneas per hour of sleep (i.e., the apnea/hypopnea index [AHI]), even in absence of weight loss, but the underlying mechanisms remain unknown (1–3). Overnight fluid shift from the legs into the neck has been indicated as a contributor to OSA (4). We have previously shown that prevention of fluid accumulation in the legs, obtained by wearing compression stockings during the day, reduces the AHI by attenuating overnight fluid shift (5, 6). Moreover, intensified diuretic therapy reduced the AHI in proportion to the decrease in overnight fluid shift (7). Fluid accumulation in the legs is promoted by sedentary living (8, 9) and is counteracted by physical activity, which activates the musculovenous pumps (8–10). We hypothesized that 1 week of two periods a day of 45 minutes of moderate-speed walking will reduce the AHI in non–severely obese sedentary subjects with moderate to severe OSA by decreasing overnight fluid shift around the pharynx. Some of the results of this study have been previously reported in the form of an abstract (11).

We consecutively selected subjects having a new diagnosis of moderate to severe OSA, defined as an AHI greater than 15, and a sedentary lifestyle, defined as not practicing physical activity regularly. Subjects were excluded if they were severely obese (body mass index [BMI], >35 kg/m^2) and if they had a current history of smoking or alcohol abuse, history of nasal or pharyngeal disease or other chronic disease, use of prescribed medications, and previously treated OSA. Seven men and one woman, middle-aged (age, 56 ± 11 years) and overweight (BMI, 28.9 ± 3.9 kg/m^2), were randomly assigned to either a 1-week walking period (consisting of walking at a rate at which they were able to keep up a full conversation, which roughly corresponds to a speed of 5 km per hour, 45 minutes twice a day, late in the morning and late in the afternoon, for 7 days, while maintaining usual activities for the rest of the time) or to a 1-week control period, after which they crossed over to the other group. Physical activity was measured over the walk and control periods using ActiGraph GT3X+ (ActiGraph LLC, Fort Walton Beach, FL) in terms of the mean number of steps taken a day during the week with and without physical activity, respectively, which reflects the daily amount of walking and is a marker of musculovenous pump activation. Polysomnography and measurement of overnight changes in legs fluid volume by bioelectrical impedance (4), in neck circumference (4), and in air volume of the pharynx from the hard palate level to the upper border of the hyoid bone by magnetic resonance imaging were performed at the end of the walk and control periods.

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