Early View

Research letter

Generalized mosaicism for *TSC2* mutation in isolated Lymphangioleiomyomatosis

Barbara Ogorek, Lana Hamieh, Kathryn Lasseter, Shefali Bagwe, Tania Machado, Carmen Herranz-Ors, Aaron R. Thorner, Anwesha Nag, Peter Gulleman, Krinio Giannikou, Lisa R. Young, Miquel Àngel Pujana, Thomas N. Darling, Souheil El-Chemaly, Joel Moss, Elizabeth P. Henske, David J. Kwiatkowski

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To the Editor,

Lymphangioleiomyomatosis (LAM) is a rare, slowly progressive pulmonary disease, causing cystic lung destruction and respiratory failure. It affects predominantly premenopausal women, and rarely men. It can occur as a sporadic condition (sporadic LAM) or in association with Tuberous Sclerosis Complex (TSC-LAM) [1]. LAM is caused by biallelic inactivation of the tumor suppressor gene *TSC2* in LAM cells, which leads to hyperactivation of mTORC1, resulting in anabolism and LAM cell proliferation [2]. Sirolimus and everolimus, mTORC1 allosteric inhibitors, have been shown to retard progression of LAM [3].

Histopathologically, LAM is characterized by the presence of nodules containing spindle-shaped LAM cells scattered throughout the lung that express smooth muscle and melanocytic markers, including α-SMA and HMB45, respectively. Similar cells proliferate on the luminal surface of the thoracic duct and other lymphatic channels, from which they break off as clusters, and are transported through the lymphatic and then venous circulation, and then the pulmonary artery, to the lung [4].

Approximately 30% of women with sporadic LAM have renal angiomyolipoma, which are benign tumors of the kidney composed of vascular, smooth muscle, and fat cells. Genetic analysis of microdissected LAM lesions from the lung and kidney angiomyolipoma of the same patient have shown identical *TSC2* mutations, indicating that they are clonal neoplasms with a common cell of origin [5].

Although high-resolution chest computed tomography shows the hallmark bilateral thin-walled cysts in patients with LAM, diagnostic uncertainty for such patients is not uncommon. Increased serum vascular endothelial growth factor-D levels (VEGF-D >800pg/ml) are common in LAM, and can be used to facilitate diagnosis. However, lung biopsy is often required for definitive diagnosis [6]. Therefore, new biomarkers for LAM are of great interest.

Plasma cell-free DNA (cfDNA) is double stranded, short (median size 150-200 base pairs in length), and thought to be derived from apoptotic or necrotic cells [7]. In patients with cancer, plasma cfDNA often contains DNA derived from cancer cells, and cfDNA analysis is now commonly performed in multiple cancer types to detect mutations that enable targeted therapy, such as epidermal growth factor receptor (EGFR) mutations for lung adenocarcinoma [8, 9]. Given the model of LAM pathogenesis with universal involvement of the lymphatics and release of LAM cell clumps into the circulation, we hypothesized that LAM cells might die in the circulation to some degree, and contribute to the pool of plasma cfDNA. Thus, analysis of cfDNA in LAM patients might identify *TSC2* mutations, and such findings might serve as both a diagnostic and prognostic marker in LAM.

We collected peripheral blood plasma from 61 sporadic LAM patients, who did not meet diagnostic criteria for TSC, using EDTA tubes (for rapid processing) or in Streck tubes (for shipment overnight). Plasma was isolated and cfDNA extracted by standard methods. Buffy coats were used for genomic DNA isolation. On average we obtained 10 ng of cfDNA per ml of plasma with median of 7ng/ml and range of 2-74 ng/ml. Targeted capture for 50 mTOR pathway genes, including the entire non-repetitive

genomic extent of *TSC2* was performed [10]. The mean coverage was 1060x (median, 1107x; range, 318-1788x). We used a custom pipeline to identify low frequency variants in *TSC1* and *TSC2* [10-12]. Candidate pathogenic Single Nucleotide Variants (SNVs) were identified based on presence in at least 3 reads, with at least one read in each orientation, and allele frequency (AF) ≥ 0.5% [10]. Candidate insertions and deletions (indels) were identified based on presence in at least 2 reads and AF ≥ 0.2%. Candidate variants were reviewed extensively, including using Integrative Genomics Viewer (IGV) and functional assessments *in silico*. All candidate variants were independently validated using amplicon MPS. For this purpose, primers flanking mutation sites were designed, and PCR amplification was performed on all available samples, including additional cfDNA samples, and blood genomic DNA. Two normal control DNA samples for each amplicon were included.

The 61 female sporadic LAM patients who were screened had median age at diagnosis of 41 years (range, 23-73, Figure 1A) with median duration of disease of 4 years (range, 1-28). Twenty-six (43%) patients had angiomyolipoma, and these tumors were bilateral in 4 (7%). Twenty-seven (44%) patients had experienced pneumothorax; 3 (5%) chylothorax; 1 (2%) hemothorax; and 2 (3%) pleural effusion. Twelve (20%) had retroperitoneal lymphangioleiomyoma. Pulmonary function tests (PFT) interpreted according to standard criteria [13] indicated that 14 (23%) patients had severe, 16 (26%) moderate, and 8 (13%) mild loss of lung function; 22 (36%) had normal lung function (for 1 (2%) patient PFT information was not available). Seven (11%), all with severe PFT, were being treated with rapalogs at the time of analysis. Serum VEGF-D was assessed in 55 of 61 (90%) patients, with a median of 755 pg/ml (range, 247-5616

pg/ml, Figure 1A). Testing was performed either as a CAP/CLIA compliant test (Cincinnati Children's Hospital Medical Center), or in a research setting using the Quantikine Human VEGF-D Immunoassay (R&D Systems; Minneapolis, MN) according to the manufacturer's instructions, while blinded to other clinical information and the genetic analyses.

A single pathogenic variant in *TSC2* (*c.2251C>T*, p.Arg751*) at allele frequency of 1.83% was identified in one LAM cfDNA sample. There were no genetic findings in any of the other cfDNA samples. Validation by amplicon MPS of multiple DNA samples from the same patient including those derived from blood, normal skin biopsy, saliva, urine and a second plasma cfDNA showed that the p.Arg751* variant was present in all samples with a median allele frequency of 2.13% ranging from 1.51% (saliva) to 3.12% (blood), indicating that the patient had generalized low level mosaicism for this *TSC2* mutation. Following this finding, this patient was seen again clinically and had a thorough detailed review of all TSC manifestations including skin exam by an expert TSC dermatologist (TD) and review of all past scans including brain MRI. The patient had a bilateral angiomyolipomas (the largest 1.8x1.3cm) (Figure 1C), retroperitoneal nodules (left iliac 1.2cm) and 10 sclerotic bone lesions. She had an elevated serum VEGF-D level (1143 pg/ml) and normal pulmonary function tests. There was no other evidence of TSC in this individual.

These findings indicate that plasma cfDNA analysis in LAM does not detect *TSC2* mutations at significant frequency, at least using the methods utilized here in which a minimum mutant allele frequency of $\geq$ 0.5% can be detected, an approach that

has been robust in multiple past studies of TSC mosaicism [11, 12, 14]. Future studies with a more sensitive assay might increase *TSC2* variant identification in LAM cfDNA.

Nonetheless, we have identified one LAM subject who does not meet TSC diagnostic criteria, who has generalized mosaicism for a pathogenic TSC2 mutation, that has previously been identified in at least 36 TSC individuals [15]. A variable phenotype, with some individuals not meeting TSC diagnostic criteria, has been reported for several rare *TSC2* missense mutations. However, to our knowledge this is the first patient identified with mosaicism for a strongly inactivating *TSC* gene mutation who does not meet diagnostic criteria for TSC. It is notable that this patient had bilateral angiomyolipoma, one of only 4 in this cohort, and multiple sclerotic bone lesions, suggesting that these clinical findings may be predictive of mosaicism for a *TSC2* mutation in sporadic LAM. This possibility requires further investigation.

Barbara Ogorek[1], Lana Hamieh[1], Kathryn Lasseter[1], Shefali Bagwe[1], Tania Machado[2], Carmen Herranz-Ors[3], Aaron R. Thorner[4], Anwesha Nag[4], Peter Gulleman[5] Krinio Giannikou[1], Lisa R. Young[5], Miquel Àngel Pujana[3], Thomas N. Darling[6], Souheil El-Chemaly[1], Joel Moss[2], Elizabeth P. Henske[1], David J. Kwiatkowski[1]*

Author Affiliations: [1]Department of Medicine, Brigham and Women's Hospital, Boston, Massachusetts, USA. [2]Pulmonary Branch, National Heart, Lung, and Blood Institute (NHLBI), USA. [3]ProCURE, Catalan Institute of Oncology (ICO), Bellvitge Institute of Biomedical Research (IDIBELL), Barcelona, Spain. [4]Center for Cancer Genome Discovery, Dana-Farber Cancer Institute, Boston, Massachusetts, USA. [5]Division of

Pediatric Pulmonary Medicine, Department of Pediatrics, Vanderbilt University School of Medicine, Nashville, Tennessee., USA. [6]Department of Dermatology, Uniformed Services University of the Health Sciences, Bethesda, Maryland, USA.

*Correspondence to: David J. Kwiatkowski, Division of Pulmonary and Critical Care Medicine and Genetics, Department of Medicine, Brigham and Women's Hospital, 20 Shattuck Street Thorn 826, Boston, MA 02115. Tel:857-307-0781; E-mail: dk@rics.bwh.harvard.edu.

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