



Generalised mosaicism for *TSC2* mutation in isolated lymphangioleiomyomatosis

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) [1]. LAM is caused by biallelic inactivation of the tumour suppressor gene *TSC2* in LAM cells, which leads to hyperactivation of mammalian target of rapamycin complex (mTORC)1, 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 characterised by the presence of nodules containing spindle-shaped LAM cells scattered throughout the lung that express smooth muscle and melanocytic markers, including smooth muscle α -actin 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].

~30% of women with sporadic LAM have renal angiomyolipoma, which are benign tumours 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 (VEGF)-D levels (>800 pg·mL⁻¹) 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 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 hypothesised 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 cfDNA per millilitre of plasma with median of 7 ng·mL⁻¹ and range of 2–74 ng·mL⁻¹. Targeted capture for 50 mammalian target of rapamycin pathway genes, including the entire nonrepetitive genomic extent of *TSC2*, was performed [10]. The mean coverage was 1060× (median, 1107×, range 318–1788×). We used a custom pipeline to identify low-frequency variants of *TSC1* and *TSC2* [10–12]. Candidate pathogenic single-nucleotide variants were identified based on presence in at least three reads, with at least one read in each orientation, and allele frequency (AF) $\geq 0.5\%$ [10].



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Analysis of plasma cell-free DNA from 61 sporadic lymphangioleiomyomatosis (LAM) patients identified generalised mosaicism for a *TSC2* mutation in one, suggesting that some sporadic LAM patients are occult generalised mosaics for *TSC2* mutations <http://bit.ly/2yLr0Ls>

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Candidate insertions and deletions were identified based on presence in at least two reads and AF $\geq 0.2\%$. Candidate variants were reviewed extensively, including using Integrative Genomics Viewer (Broad Institute, San Diego, CA, USA) and functional assessments *in silico*. All candidate variants were independently validated using amplicon massively parallel sequencing (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 years) (figure 1a) with median duration of disease of 4 years (range 1–28 years). 26 (43%) patients

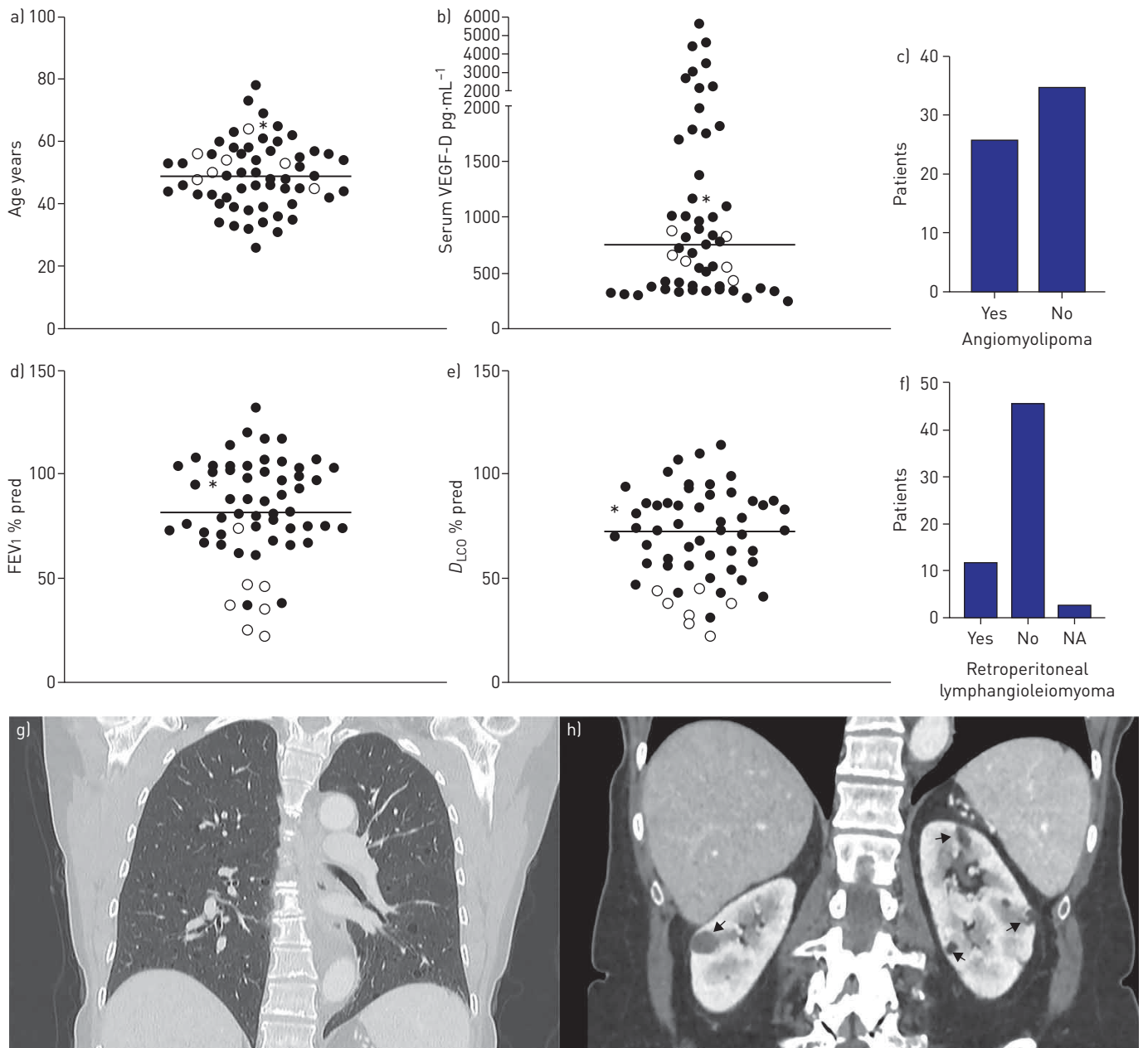


FIGURE 1 Clinical characteristics of lymphangioleiomyomatosis (LAM) cohort and patient with generalised *TSC2* mosaicism. a) Current age distribution for 61 LAM patients (median 49 years). b) Serum vascular endothelial growth factor (VEGF)-D concentrations for 55 out of 61 LAM patients (median 755 ng·mL⁻¹). c) Number of patients with angiomyolipoma. d) Forced expiratory volume in 1 s (FEV₁) for 60 of 61 LAM patients (median 82% pred). e) Diffusing capacity of the lung for carbon monoxide (*D*_{LCO}) for 45 of 61 LAM patients (median 76% pred). f) Number of patients with retroperitoneal lymphangioleiomyoma. a, b, d and e) Median is indicated by the horizontal line. Empty circles correspond to individuals treated with rapamycin. g) Chest and h) abdominal computed tomography images (coronal view) of mosaic patient demonstrating lung cysts (g) and bilateral kidney angiomyolipoma (arrows in h). NA: not available. *: patient with generalised *TSC2* mosaicism.

had angiomyolipoma and these tumours were bilateral in four (7%). 27 (44%) patients had experienced pneumothorax; three (5%), chylothorax; one (2%), haemothorax; and two (3%), pleural effusion. 12 (20%) had retroperitoneal lymphangiomyoma. Pulmonary function tests (PFT) interpreted according to standard criteria [13] indicated that 14 (23%) patients had severe, 16 (26%) moderate and eight (13%) mild loss of lung function; 22 (36%) had normal lung function (for one (2%) patient, PFT information was not available). Seven (11%), all with severe PFT, were being treated with rapalogues at the time of analysis. Serum VEGF-D was assessed in 55 (90%) out of 61 patients, with a median of 755 pg·mL⁻¹ (range 247–5616 pg·mL⁻¹) (figure 1b). Testing was performed either as a College of American Pathologists/Clinical Laboratory Improvement Amendments-compliant test (Cincinnati Children's Hospital Medical Center, Cincinnati, OH, USA) or in a research setting using the Quantikine Human VEGF-D Immunoassay (R&D Systems, Minneapolis, MN, USA) according to the manufacturer's instructions, while blinded to other clinical information and the genetic analyses.

A single pathogenic variant of *TSC2* (c.2251C>T, p.Arg751*) at AF 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 generalised, 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 examination by an expert TSC dermatologist (T.N. Darling) and review of all past scans, including brain magnetic resonance imaging. The patient had a bilateral angiomyolipomas (the largest 1.8×1.3 cm) (figure 1h), retroperitoneal nodules (left iliac 1.2 cm) and 10 sclerotic bone lesions. She had an elevated serum VEGF-D level (1143 pg·mL⁻¹) and normal PFT results. 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 utilised here, in which a minimum mutant AF ≥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 did not meet TSC diagnostic criteria, who had generalised mosaicism for a pathogenic *TSC2* mutation that has previously been identified in at least 36 TSC individuals (www.lovd.nl). 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 did not meet diagnostic criteria for TSC. It is notable that this patient had bilateral angiomyolipoma, one of only four 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 Ogórek¹, Lana Hamieh¹, Kathryn Lasseter¹, Shefali Bagwe¹, Tania Machado², Carmen Herranz-Ors³, Aaron R. Thorner⁴, Anwesha Nag⁴, Peter Gulleman⁵, Krinio Giannikou¹, Lisa R. Young⁵, Miquel Àngel Pujana³, Thomas N. Darling⁶, Souheil El-Chemaly¹, Joel Moss², Elizabeth P. Henske¹ and David J. Kwiatkowski¹

¹Dept of Medicine, Brigham and Women's Hospital, Boston, MA, USA. ²Pulmonary Branch, National Heart, Lung, and Blood Institute (NHLBI), Bethesda, MD, USA. ³ProCURE, Catalan Institute of Oncology (ICO), Bellvitge Institute of Biomedical Research (IDIBELL), Barcelona, Spain. ⁴Center for Cancer Genome Discovery, Dana-Farber Cancer Institute, Boston, MA, USA. ⁵Division of Pediatric Pulmonary Medicine, Dept of Pediatrics, Vanderbilt University School of Medicine, Nashville, TN, USA. ⁶Dept of Dermatology, Uniformed Services University of the Health Sciences, Bethesda, MD, USA.

Correspondence: David J. Kwiatkowski, Division of Pulmonary and Critical Care Medicine and Genetics, Dept of Medicine, Brigham and Women's Hospital, 20 Shattuck Street Thorn 826, Boston, MA 02115, USA.
E-mail: dk@rics.bwh.harvard.edu

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