Mutation spectrums of TSC1 and TSC2 in Chinese women with lymphangioleiomyomatosis (LAM)

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Abstract

The aim of our study was to elucidate the landscapes of genetic alterations of TSC1 and TSC2 as well as other possible non-TSC1/2 in Lymphangioleiomyomatosis (LAM) patients. Sixty-one Chinese LAM patients’ clinical information was collected. Tumor biopsies and matched leukocytes from these patients were retrospectively analyzed by next generation sequencing (NGS), chromosomal microarray analysis (CMA), and multiplex ligation-dependent probe amplification (MLPA). Eighty-six TSC1/2 variants were identified in 46 of the 61 LAM patients (75.4%) in which TSC2 and TSC1 variants were 88.37% and 11.63% respectively. The 86 variants are composed of (i) 52 single nucleotide variants (SNVs) (including 30 novel variants), (ii) 23 indels (including 21 deletions, and 2 insertions), (iii) a germline duplication of exon 31–42 of TSC2, and (v) 9 regions with copy-neutral loss of heterogeneity (CN-LOHs) present only in the LAM patients with single TSC1/2 mutations. Sixty-one non-TSC1/2 variants in 31 genes were identified in 37 LAM patients. Combined applications of different techniques are necessary to achieve maximal detection rate of TSC1/2 variants in LAM patients. Thirty novel TSC1/2 variants expands the spectrum of TSC1/2 in LAM patients. Identification of 61 non-TSC1/2 variants suggests that alternative genes might have contributed to the initiation and progression of LAM.
Introduction

Lymphangioleiomyomatosis (LAM) is a rare, low-grade, destructive, and metastasizing neoplasm that mainly affects women, usually during their childbearing years, and occasionally affects men [1–3]. Clinical features are characterized by progressive cystic destruction of the lung parenchyma, recurrent pneumothorax, development of fluid-filled lymphatic cystic structures (lymphangioleiomyomas), chylothorax, and abdominal angiomyolipomas. Clinical diagnosis can be routinely achieved based on symptoms and results from radiological scanning, pathological biopsies and subsequent immuno-histochemical staining [1,4–7].

LAM is known to be caused by mutations in the tuberous sclerosis complex (TSC) genes, TSC1 located on chromosome 9q34 encoding hamartin or TSC2 located on chromosome 16p13 encoding tuberin [8–10]. Mutations in both hamartin and tuberin lead to inactivation of the tuberin-hamartin complex, resulting in increased activity of a kinase known as the mammalian target of rapamycin (mTOR) leading to abnormal proliferation of smooth muscle-like cells (LAM cells), the underlying causes of a variety of pathological findings.

LAM can occur sporadically (S-LAM) or in association with TSC (TSC-LAM), an autosomal dominant syndrome characterized by multisystem growth of hamartoma (such as, lung, brain, kidney, heart, retina and skin) as well as neurological features (such as, seizures, autism, and intellectual disabilities). Consistent with the Knudson’s “two-hit” theory for explaining the tumorigenesis in which usually through a mutation in one allele coupled with loss of the remaining wild-type allele [11,12], S-LAM develops arising from two somatic mutations in TSC1/2 while TSC-LAM have one germline mutation and one acquired mutation [13,14]. However, two hits (mutations) in TSC1/2 are not always observed in patients with definite diagnosis of LAM, and the underlying reasons haven’t been comprehensively investigated yet.

In this study, we systematically investigated 61 Chinese female LAM patients focusing on unveiling the landscapes of genetic alterations of TSC1/2 as well non-TSC1/2 in their genomes. A comprehensive landscape of TSC1/2 genetic alterations leading to the tumorigenesis of LAM were identified, including single-nucleotide variants (SNVs), small insertions or deletions (indels), copy number variants (CNVs), and regions of copy-neutral loss-of-heterozygosity (CN-LOH), with more than one-third of the variants being novel mutations. Meanwhile, we identified a long list of variants in non-TSC1/2 genes, and proposed additional mechanisms participating in the initiation and progression of S-LAM.

Materials and methods

Patients

Seventy-six Chinese patients with definite diagnosis of LAM disease were initially enrolled and 61 of them were retrospectively analyzed in this project. All of these patients were diagnosed, treated, and followed up in the Guangzhou Institute of Respiratory Health from April of 2011 to Dec. of 2017 according to the clinical guidelines [2,15]. Patients’ general information, disease history, family history, radiological findings, pathological and relevant laboratory testing results were collected from their medical records and summarized in S1 and S2 Tables. For this retrospective study, the Medical Ethics Committee of the First Affiliated Hospital of Guangzhou Medical University waived the requirement to obtain consent from patients included in the research project on April 22, 2019, with approval number:2019-K-18, considering the fact that all data were retrospectively analyzed anonymously.
Specimens and DNA extractions

Ninety-nine formalin-fixed paraffin-embedded (FFPE) specimens were collected from the 76 LAM patients. These FFPE specimens were prepared as part of routine clinical care from different biopsies with the majority of them from lung and the some from kidney, retroperitoneal, pelvic cavity, and uterus (Fig 1, Table 1).

Before DNA extraction, biopsy specimens were reviewed by at least one certified clinical pathologist to assess the presence of LAM cells. DNAs from these biopsy specimens were isolated using GeneRead DNA FFPE kit (QIAGEN, Hilden, GER). After evaluation of the DNA qualities, 75 of the 99 biopsy specimens from 61 of the 76 LAM patients were qualified for further NGS analysis, leaving 24 specimens from 15 LAM patients being excluded from this study. The 61 patients with 75 biopsy specimens are composed of 49 patients with a single specimen (40 patients with single lung specimens, 7 patients with single kidney specimens, one patient with a single retroperitoneal specimen, and one patient with a single pelvic cavity specimen), 11 patients with two separate specimens (7 patients with 2 lung specimens, 2 patients with 2 kidney specimens, 1 patient with 1 lung specimen plus a kidney specimen, and 1 patient with 1 lung specimen plus a uterus specimen), and 1 patient with 4 specimens (3 lung specimens plus a retroperitoneal specimen). We could also extract leukocyte DNAs from 44 of the 61 LAM patients using QIAamp DNA Mini Kit (QIAGEN, Hilden, GER) (Fig 1).

Table 1. Tissue biopsy specimens obtained from the 61 LAM patients.

| Specimens (number of patients) | Sampling spots       | Number of patients | Number of specimens |
|-------------------------------|----------------------|--------------------|---------------------|
| 1 specimen (49)               | lung                 | 40                 | 40                  |
|                               | retroperitoneal      | 1                  | 1                   |
|                               | pelvic cavity        | 1                  | 1                   |
|                               | kidney               | 7                  | 7                   |
| 2 specimens (11)             | 2 specimens from lungs | 7                 | 14                  |
|                               | 1 from lung and 1 from kidney | 1               | 2                   |
|                               | 1 from lung and 1 from uterus | 1              | 2                   |
|                               | 2 specimens from kidney | 2                | 4                   |
| 4 specimens (1)              | 3 from lung and 1 from retroperitoneum | 1             | 4                   |
| Total                         |                      | 61                 | 75                  |

https://doi.org/10.1371/journal.pone.0226400.t001
Target capture sequencing

A custom gene panel (S3 Table) were designed for sequencing the genomic DNA (gDNA) extracted from the biopsies. The gene panel encompasses 76 key genes including TSC1 and TSC2 related to tumorigenesis in a variety of malignant tumors. gDNA library preparations were performed with KAPA Hyper Plus Kit, (Kapa Biosystems, Wilmington, USA). The library mixtures were performed by a hybrid capture-based procedure using xGen Lockdown Reagent (Integrated DNA Technologies, Coralville, USA). The captured libraries were sequenced on an Illumina Miseq or Nextseq500 sequencer (Illumina, San Diego, USA) for 150 cycles of paired-end reads with mean target coverage >1000x and minimum depth of coverage ≥200x. Following sequencing, fastq files were generated with Illumina software, sequencing reads were aligned to the GRCh37/hg19 assembly with BWA-MEM, and variants were filtered, called, and annotated using GATK, Pisces and ANNOVAR, respectively. Variant allelic fractions (VAF) of somatic variants (SNVs or indels) down to 1.0% were achieved, and a total read count ≥200 and a variant read count ≥5 were set up as the minimum numbers required in this study. The clinical significance of the germline and somatic variants were assessed according to the published guidelines and related database LOVD2.0 [16–20]. Data have been deposited at the European Genome-phenome Archive, which is hosted by the European Bioinformatics Institute, under study accession number EGAS00001003534.

Sanger sequencing

Sanger sequencing for DNAs extracted from leukocytes was used to characterize some genetic variants identified by next generation sequencing (NGS) from tumor DNAs in which it's difficult to determine whether these variants were germline-originated or somatically-mutated in TSC1/2. Based on our validated pipelines, a variant with VAF ≥30% is considered somatic while a variant with 30% ≤ VAF ≤ 70% is considered germline for both variants identified in TSC1/2 and non-TSC1/2. To further validate the origins of the variants in TSC1/2, Sanger sequencing was applied using DNAs extracted from leukocytes in 21 patients with 26 TSC1/2 variants identified in their tumor DNAs by NGS with VAF ranging from 10% to 75%. Sanger sequencing were performed on ABI-3730xl system (Thermo Fisher Scientific, Carlsbad, USA).

Chromosomal microarray analysis (CMA)

Biopsied DNA samples from 24 LAM patients were performed by CMA method to detect CNVs and CN-LOH according to the manufacturer’s instruction (Thermo Fisher Scientific, Carlsbad, USA). The CMA platform applied is composed of 550,000 non-polymorphic CNV probes and more than 200,000 Single nucleotide polymorphism (SNP) probes with an average resolution of 100 kb. This SNP array platform can detect both CNVs and CN-LOH. All data were visualized and analyzed with the Chromosome Analysis Suite (ChAS) software. The analysis was based on the reference genome of GRCh37/hg19 assembly. The clinical significance of CNVs and CN-LOHs were assessed according to the published guidelines [21,22].

Multiplex Ligation-dependent probe amplification (MLPA)

Leukocyte DNAs extracted from the 44 LAM patients were tested by MLPA to identify CNVs of TSC1/2 with SALSA MLPA probe-mix P124-C1 (TSC1) and P046-C1 (TSC2) according to the manufacturer’s instruction (MRC-Holland; Amsterdam, The Netherlands).
Statistical methods

Several statistical methods were used for analyzing the data in this study. Fisher’s exact test was performed for comparing the status of CN-LOHs in LAM patients carrying different numbers of TSC1/2 variants. Chi-square testing was used for comparing the clinical information and genetic results of TSC1/2 in the TSC-LAM and S-LAM. One-way ANOVA test was used for comparing novel and previously identified in TSC1/2 variants. All tests were two-tailed, with statistical significance defined as $P < 0.05$. These statistical analyses were performed with Graphpad Prism 6.0 software and IBM SPSS Statistics 22.

Results

Clinical, imaging, pathological, and biochemical findings

Of the 61 patients, 10 (16.4%) of them were diagnosed as TSC-LAM according to the recommendations from the International Tuberous Sclerosis Complex Consensus Group [23,24], and 5 (8.2%) of them had a family history of TSC. Concurrent presence of lung adenocarcinoma previously diagnosed in a TSC-LAM patient (ID052) was validated (S1 Fig).

The most common clinical manifestations in the 61 LAM patients include breathlessness in 41 (67.2%) patients (9 TSC-LAM, 32 S-LAM), spontaneous pneumothorax in 34 (55.7%) (5 TSC-LAM, 29 S-LAM), and chylothorax in 3 (4.9%) patients (0 TSC-LAM, 3 S-LAM). Chest Computer Tomography (CT) scans identified diffuse cystic features and multiple bullies in all the 61 patients including 6 cases (9.8%) in grade I (0 TSC-LAM, 6 S-LAM), 27 cases (44.3%) in grade II (5 TSC-LAM, 22 S-LAM), and 28 cases (45.9%) in grade III (5 TSC-LAM, 23 S-LAM). Abdominal CT scans found extrapulmonary involvements in LAM patients including renal angiomyolipoma in 29 (47.5%) patients (8 TSC-LAM, 21 S-LAM), pelvic uterine lymphangioleiomyomas in 13 (21.3%) patients (0 TSC-LAM, 13 S-LAM), liver hamartoma in 11 (18%) patients (3 TSC-LAM, 8 S-LAM), and retroperitoneal lymphangioleiomyomas in 9 (14.8%) patients (1 TSC-LAM, 8 S-LAM). It is interesting to notice that the percentage (80.0%) with renal angiomyolipomas in TSC-LAM is significantly different from that (41.2%) in S-LAM ($P < 0.05$).

The pathological findings showed characteristic features of LAM in all the analyzed tissues, such as cystic lesions in pulmonary tissues, multiple immature smooth muscle cells and perivascular epithelial cells in pulmonary and other tissues. Multiple immunohistochemical staining was performed showing positive SMA and HMB45 results in all 61 (100%) cases as well as positive ER (9 TSC-LAM, 38 S-LAM) and PR (8 TSC-LAM, 39 S-LAM) results in 47 (77%) cases respectively.

Serum VEGF-D levels were determined by enzyme-linked immunosorbent assay in all patients in this study at their first visits pursuing diagnosis and treatments. The average serum VEGF-D level were 1,689 pg/ml from TSC-LAM patients and 1,570 pg/ml from S-LAM patients, respectively. Thirty-eight (62.3%) patients had serum VEGF-D levels >800 pg/ml as supporting evidence for the diagnosis of LAM disease in these patients.

Genetic variants in the TSC1 and TSC2 genes in the LAM patients

A combined use of methods (NGS, Sanger sequencing, CMA, and MLPA) identified a total of 86 clinically significant genetic variants of TSC1/2 in 46 of the 61 LAM patients, with the remaining 15 patients showing negative results, representing an overall positive detection rate of 75.4% (46/61) in which TSC2 and TSC1 variants were 88.37% and 11.63% respectively (Fig 1, S4 Table). These results were further organized and analyzed in different ways in following paragraphs.
Based on the testing methods used, NGS identified 75 of the 86 variants in 75 biopsies from 44 patients, including 52 single nucleotide variants (SNVs) (9 in TSC1 and 43 in TSC2), 21 small deletions, and 2 small insertions in TSC2 (Fig 2A and 2B). Among the 75 variants, 45 of them were previously documented in literatures and/or public databases, leaving 30 of the variants as novel findings consisting of 13 SNVs (2 in TSC1 and 11 in TSC2) and 17 indels (0 in TSC1 and 17 in TSC2) (S4 Table, Fig 3A and 3B), there were no significant differences in the distribution of substitution between novel and previously identified in TSC1/2 (P > 0.05); MLPA identified a germline duplication of exon 31–42 in TSC2 in patient ID180; and CMA identified a 2.68 Mb somatic duplication with TSC2 involved in patient ID160 and 9 CN-LOHs in 9 out of the 24 patients with surplus samples available for testing from the 61 LAM patients (S4 Table, Fig 4A and 4B). Our results showed that all 9 CN-LOHs were present in the LAM patients with single TSC1/2 mutations but absent in the LAM patients with 2 or more TSC1/2 mutations or without detectable TSC1/2 mutations (S5 Table) (P < 0.05). Specifically mentioned here that, in addition to identifying two mutations in TSC2, CMA detected complex genomic abnormalities involving multiple chromosomes in patient ID052 with concurrent diagnosis of lung adenocarcinoma (S2 Fig). However, we didn’t find similar chromosomal alterations in other LAM patients in this study.

If a variant was germline-originated, the ACMG guideline was used to determine its clinical significance [18]. If a variant was somatic, the guideline published by Li et al. was used to determine its clinical significance. Meanwhile, Leiden Open Variation and ClinVar Database were highly referenced, CNV and CN-LOH events affecting TSC1 and TSC2 were assumed detrimental to gene function. Based on the numbers of TSC1/2 variants identified in each patient, the 46 patients with 86 significant variants in TSC1/2 could be categorized into 3 groups: 1) eight patients were found to carry single variants of TSC1/2 including germline variants in 2 cases (ID140 with a SNV of TSC2/p.Arg1459/Pathogenic and ID180 with a duplication of exon 31–42 in TSC2/Likely Pathogenic, however, no obvious symptoms of TSC and no angiomyolipomas or sclerotic bone lesions complications in this patient whom without family history.) and somatic variants in 6 cases (ID030, ID076, ID099, ID060, ID115 and ID162) with lung specimens tested in 7 of the 8 patients except in patient ID115 with a kidney specimen being tested; 2) thirty-six patients were found to carry 2 variants of TSC1/2 including 1 patient (ID148) with a germline mutation (TSC1/p.Met879Thr/VUS) plus a somatic mutation (TSC2/p.Arg1438Gln/Tier III), 1 patient (ID131) with a germline mutation (TSC2/c.2220+2_2220+9del/Likely Pathogenic) plus a somatic mutation (TSC2/p.Ser1252Leu/Tier III), 1 patient (ID052) with a germline mutation (TSC2/p.Trh603Ser/VUS) plus a CN-LOH (TSC1), 1 patient (ID187) with 2 VUS germline mutations occurred in the same allele of TSC1 (p.Arg517Trp/p.Arg484Gly), 5 patients (ID015-uterus, ID023-lung1, ID058-lung1&amp;2, ID137-lung, ID153-lung) carrying 2 somatic
mutations with one of them in TSC1 and the other in TSC2 (Of the 5 patients carrying both TSC1/2, all of the 5 TSC2 variants were classified as Tier I or Tier II while all of the TSC1 variants were classified as Tier III), 8 patients with a somatic (TSC2/Tier I or Tier II) plus a
CN-LOH (TSC2/Tier I), and 19 patients carrying 2 somatic mutations in the same gene of TSC2; 3) two patients were found to carry 3 mutations including patient ID152 and ID154 carrying a germline (TSC2) plus 2 somatic mutations (TSC2) respectively, the germline mutations in patient ID152 (TSC2/p.Ser1799del) and ID154 (TSC2/p.Thr1260Met) were classified as VUS while the two TSC2 somatic mutations were considered to be Tier I or Tier II variants. Detailed information about these genetic variants was listed in S4 Table.

Based on the genetic findings in TSC1/2, correlations between genotypes and clinical diagnosis (TSC-LAM or S-LAM) for the 61 patients were analyzed. Eleven of the 86 TSC1/2 variants were determined to be germline mutations (4 in TSC1 and 7 in TSC2) while the remaining 75 variants occurred somatically. The 11 germline mutations were present in 10 patients including 1 patient (ID187) carrying 2 mutations in one allele. Eight and 2 of the 10 patients were clinically diagnosed as TSC-LAM and S-LAM respectively. A total of 9 germline mutations were detected in 8 of the 10 TSC-LAM patients, including 2 pathogenic mutations in patients ID140 (TSC2/p.Arg1459*) and ID130 (TSC2/p.Trp1740*), 1 Likely Pathogenic mutations in ID131 (TSC2/c.2220+2_2220+9del), and 6 VUS: ID052 (TSC2/p.Thr603Ser), ID058 (TSC1/p.Asn198Ser), ID148 (TSC1/p.Met879Thr), ID152 (TSC1/p.Ser1799del) and ID187 (TSC1/p.Arg517Trp, TSC1/p.Arg484Gly). In addition, in 1 (ID140) of the 10 patients

Fig 4. Detection of CN-LOHs in the LAM patients. (A) CN-LOHs involving TSC1 in LAM patients. (B) CN-LOHs involving TSC2 in LAM patients. CN-LOH events were always considered somatic, and co-occurred with a somatic mutation identified in the same tissues in our study.

https://doi.org/10.1371/journal.pone.0226400.g004
with clinical diagnosis of TSC-LAM, only a germline mutation in TSC2 (first hit) was found but failed to find the second somatic mutation (second hit) in her genome. Conversely, no germline variant was detectable in the 2 patients with clinical diagnosis of TSC-LAM although somatic mutations were found in both of them, patient ID076 carrying a somatic nonsense mutation in TSC1 and patient ID183 carrying 2 somatic mutations in TSC2 (a deletion of exon 34 and an insertion of exon 41 in TSC2 from two separate kidney biopsies and both of them causing frameshift mutations). Taken together, all the 10 patients (100%) with clinical diagnosis of TSC-LAM were found to carry TSC1/2 mutations while only 36 of the 51 (70.6%) patients with clinical diagnosis of S-LAM were found to carry TSC1/2 mutations, with a statistically significant difference between the positive detection rates in these two subgroups (P<0.05).

Genetic variants in the non-TSC1/2 genes in LAM patients

In addition to the 86 significant genetic variants identified in TSC1 and TSC2, 61 variants in 31 non-TSC1/2 genes related to tumorigenesis were found in the 37 LAM patients, including 1 frame-shift deletion (ID130), 1 in-frame insertion (ID054), 3 nonsense mutations (ID086, ID150, and ID162), 2 splicing mutations (ID015 and ID135), and 54 missense mutations (S6 Table, Fig 5). According to the published guidelines and related database LOVD2.0 [16–20], the 61 non-TSC1/2 variants were further categorized into 3 groups based on their clinical significance, including 6 pathogenic (Tier I), 6 likely pathogenic (Tier II), and 49 VUS (Tier III) (S6 Table). Of the 12 variants classified as pathogenic or likely pathogenic, their allele frequencies were not found in the public population databases and were ≤0.01% present in our internal database deriving from more than 30,000 Chinese individuals.

Based on their cell origins, the 61 non-TSC1/2 variants were grouped into 20 germline and 41 somatic mutations. The 20 non-TSC1/2 germline variants were mutated in 15 genes in 18 LAM patients, with 3 genes mutated in at least 2 patients, including ATRX in 4, MYCN in 2, and PGR in 2. Two patients carried 2 non-TSC1/2 germline variants, patient ID105 with mutations in ATRX and BCL2 and patient ID142 with mutations in MYCN and RET (S7 Table). The allele frequency of the 20 non-TSC1/2 germline variants in this cohort are significantly higher than their frequencies of ≤0.01% present in our internal database deriving from more than 30,000 Chinese individuals and publicly available databases (P<0.05), The variants found in patient ID162 (TP53/p.Gln52*5) and ID130 (MYCN/p.Asp264Glufs*53) were considered Likely Pathogenic and the others were considered VUS.

Forty-one non-TSC1/2 somatic variants in 24 genes were found in 22 LAM patients and the mutations per patient ranged from 2 to 6. The allele frequencies of the somatic variants were either not found or ≤0.2% present in the public population databases (1000g, ExAC, gnomAD, etc.). Their clinical significances were classified according to published guidelines as described above. We found 6 Tier I mutations in 4 patients (ID025/kidney/EGFR/p.Leu858Arg and kidney/TP53/p.Arg175His, ID054/lung/FLT3/p.Pro606_Phe612dup, ID087/lung/CTNNB1/p.Ser45Phe and KRAS/p.Gly12Val, ID150/lung/RB1/p.Arg556*). Three of the 4 patients were found without significant variants in TSC1/2. Besides, 4 Tier II mutations were found in 4 patients (ID015/uterus/ATRX/c.4809+1G>A, ID086/lung/ATM/p.Gln2881*, ID115/kidney/FGFR1/p.Asn546Lys, ID135/lung/ATRX/c.6218-1G>A), and all of the other 31 mutations were considered to be Tier III variants (S8 Table). Of them, eight non-TSC1/2 genes were mutated in at least 2 LAM patients, RICTOR in 6, ATRX in 5, ERBB2 in 3, RET in 3, ATM in 2, BRAF in 2, FGFR2 in 2, and SMAD4 in 2. All the 6 RICTOR variants observed in this study were somatic while none of them was germline-generated (P<0.05). One (ID135) of the 15 LAM patients without detectible TSC1/2 variants carries two somatic variants in ATRX, and
currently it is unknown whether they are on the same allele or on different alleles. Meanwhile, a recurrent variant \( ATRX/p.\text{Arg907Gln} \) were present in both patients ID087 and ID183 respectively.

**Fig 5. Distributions of cases carrying non-TSC1/2 variants identified in this study.**

https://doi.org/10.1371/journal.pone.0226400.g005
Discussion

According to the Knudson’s “two-hit” theory [11,12], TSC-LAM is supposed to carry one germline mutation (first hit) and an additional acquired mutation (second hit) while S-LAM is supposed to carry two acquired mutations in TSC1/2 [13,14]. In the current study, although only 24.6% (15/61) of the LAM patients were absent of pathogenic or likely pathogenic TSC1/2 variants in their genomes, 47.5% (29/61) of them was considered as lack of detectable biallelic inactivation of TSC1/2, including 8 patients with single pathogenic or likely pathogenic TSC1/2 variants, 6 patients with one variant in TSC1 and the other in TSC2, and 15 patients without any detectable clinically relevant variants in TSC1/2. Based on the results from ours and others, we think the reasons leading to lack of detectable TSC1/2 mutations in some LAM patients could be categorized into two groups: technical reasons in which the methods used failed to identify the TSC1/2 mutations present in LAM patients and biological reasons in which mutations in some non-TSC1/2 genes, independently or synergizing with TSC1/2, might have contributed to tumorigenesis of S-LAM.

Technical improvements required to maximize the detection rate for TSC1/2 mutations

Currently, no single test method could identify all genetic alterations. For example, of the 86 variants involving TSC1/2 identified in this study, the target NGS identified 75 SNVs and indels, CMA identified 9 CN-LOHs and a duplication, and MLPA identified a duplication, emphasizing the significance of combining different genetic methods for achieving maximal detection rate of TSC1/2 mutations in LAM patients. It is interesting to notice that all 9 CN-LOHs were present in the LAM patients with single TSC1/2 mutations, but absent in the LAM patients with 2 or more TSC1/2 mutations or without detectable TSC1/2 mutations (S5 Table) (P<0.05), suggesting that mitotic formation of somatic CN-LOHs involving TSC1/2 is a common mechanism leading to biallelic inactivation of TSC1/2 as the underlying cause of LAM.

The VAF of a mosaic mutation in TSC1/2 might be present, but lower than the cutoff value (1%) of sequencing reads set in our study, requiring more sensitive sequencing method to unveil them. Alternatively, instead of improving the sensitivity in a testing method, Laser Capture Microdissection (LCM) techniques, an approach to enriching the LAM cells in the biopsy tissues could reach relatively higher detection rates by picking up some mutants of low sequencing reads [25,26];

Mutations located within intronic regions of TSC1/2 were not covered by our target NGS and if the mutations did have occurred and might have been missed by our methods. Tyburczy et al. reported that intronic mutations in TSC1/2 might explain some of the previously unidentified TSC1/2 mutations in TSC patients [27]. Currently, we don’t have enough DNA to further test whether some mutations might be present in the non-coding regions of TSC1/2 in the patients. Thus, in the future, it is ideal to sequence whole genes covering both exonic and intronic regions of TSC1 and TSC2 [27,28];

Further evidence required to elucidate the associations between mutations in some non-TSC1/2 genes and tumorigenesis of LAM

In the current study, all the 10 patients with clinical diagnosis of TSC-LAM were found to carry TSC1/2 mutations and 8 of them carried biallelic inactivation of TSC1/2, suggesting that it is less like that a non-TSC1/2 tumor suppressor gene might have participated in the initiation of TSC or TSC-LAM, consistent with the opinion from Lam HC et al. that “the possibility of a
third germline TSC-causing gene is unlikely” in patients with TSC [26,27,29]. Lam HC et al.’s opinion was based on multiple studies that the majority of this kind of patients have either a genetically mosaic form or an intronic mutation of TSC1/2 that could not have been detected using conventional screening methods. In contrast to the fact that all of the 10 patients with clinical diagnosis of TSC-LAM were found to carry TSC1/2 mutations, only 36 of the 51 (70.6%) patients with clinical diagnosis of S-LAM were found to carry TSC1/2 mutations (P<0.05), indicating some non-TSC1/2 mutations might have participated in the initiation of S-LAM. Interestingly, 6 somatic mutations in the RICTOR gene were identified in 6 S-LAM patients separately in this study and somatic RICTOR mutations related to stomach and intestine cancers were documented in the COSMIC database. The RICTOR (rapamycin-insensitive companion of mTOR) gene encodes a core component of the mTOR complex-2 (mTORC2) and recent findings suggested that high mTORC2 activity may play a role in the pathogenesis of LAM [10,30]. Apparently, the functional significance of these RICTOR mutations need further investigation. Another interesting finding in the current study is that 9 ATRX variants (4 germline and 5 somatic variants) were identified, representing the most common non-TSC1/2 variants in the 61 LAM patients, including one (ID135) of the 15 LAM patients without detectible TSC1/2 variants but with two somatic variants of ATRX, and a recurrent variant (ATRX/p.Arg907Gln) present in both patients ID087 and ID183 respectively. ATRX is a tumor suppressor gene located on chromosome X and biallelic inactivation of this gene through mutations or chromosome X inactivation is associated with tumorigenesis in a variety of tumors. ATRX encodes a protein required for suppression of telomeric repeat-containing RNA expression [31]. The underlying mechanism linking ATRX mutations and initiation and progression of LAM diseases is unknown currently.

Genetic and functional studies required to characterize the correlations between TSC1/2 variants and phenotypes of LAM

Interaction of trans-heterozygous TSC1/2 mutations between a mutated TSC1 and a mutated TSC2 might have contributed to tumorigenesis in the 6 patients with one variant in TSC1 and the other in TSC2 in this study. Similar cases were previously reported in the tumor tissues of patients with TSC [32]. Uhlmann EJ reported that Tsc1 +/-; Tsc2 +/- compound heterozygous mice show potential epistatic interaction between monoallelic mutation of the two genes [33,34];

Among the 36 patients with clinical diagnosis of S-LAM, patient ID180 carries a novel germline duplication of exon 31–42 in TSC2 without clinical detectable symptom of TSC. Similarly, Patient ID154 with a diagnosis of S-LAM carries 2 TSC2 variants, a germline variant identified in the blood and 2 somatic variants in her lung tissue. Possible explanation is that the symptoms of TSC in these patients were too mild to be observed routinely. Similar results were reported before, for example, Sato et al identified a novel TSC1 germline mutation in 1 (4.5%) of the 22 S-LAM patients [35]. These results lead us to speculate that some of the patients with clinical diagnosis of S-LAM might carry atypical germline mutation of TSC1/2, suggesting that genetic testing for TSC1/2 is critical to the clarification of a clinical diagnosis of LAM disease.

In patient ID187 with a diagnosis of TSC-LAM, NGS identified 2 germline mutations in the exon 15 of TSC1 in cis (on the same allele) and both were categorized as Tier III variants (VUS). Theoretically, it is less likely to have two causing mutations occurring on the same allele and both them are driver mutations causing initiation of tumorigenesis. Thus, it is reasonable to speculate that at least one of the two germline mutations may not have effect on the initiation of TSC-LAM in this patient. This speculation is probably applicable to some of the
other VUSs present in TSC1/2 identified in this study. Thus, further studies are needed to
demonstrate the biological functions of the VUSs and their association to the formation of
LAM.

Limitations of the current study
We acknowledge that there are some limitations in this study, including: 1) published results
showed that a third of the TSC1/2 mutations in patients with TSC were inherited and two
thirds occurred de novo [36–38]. Lack of pedigree information in some patients carrying
germine mutations of TSC1/2 restrained us to determine whether the germine mutations
might have inherited from one of the parents or occurred de novo. 2) Limited quantity of DNA
samples in some patients restrained us to implement further genetic testing; 3) Although some
identified novel variants of TSC1/2 were considered to be the likely causes of S-LAM in these
patients, further investigations about these variants are required to determine their abnormal
functions and pathogenicity underlying the formation of LAM; and 4) the non-TSC1/2
somatic mutations identified in the LAM patients need to be further investigated about their
association to the tumorigenesis of LAM.

Conclusion
A wide spectrum of genetic alterations in TSC1/2 identified in the cohort supports clinical
applications of a combined use of different techniques in order to achieve maximal molecular
detection rate for LAM patients. Thirty novel variants in TSC1/2 identified contributes to the
TSC1/2 data resources underlying the cause of LAM, valuable for the diagnosis and subsequent
treatment of the disease. Identification of a long list of non-TSC1/2 somatic mutations identified in the LAM patients need to be further investigated about their association to the tumorigenesis of LAM.

Supporting information
S1 Fig. Chest CT scans of patient ID052.
(DOC)

S2 Fig. Complex genomic abnormalities involving multiple chromosomes in patient ID052
by CMA.
(DOC)

S1 File. The details of supplementary figures.
(DOC)

S2 File. The details of supplementary tables.
(XLSX)

S1 Table. Clinical parameters of the 61 LAM patients.
(XLSX)

S2 Table. Baseline demographic and clinical characteristics of the patients.
(XLSX)

S3 Table. Custom gene panel designed for target sequencing.
(XLSX)
S4 Table. Genetic variants in TSC1/2 in the LAM patients.
(XLSX)

S5 Table. Relevant statistical details in this study.
(XLSX)

S6 Table. Total genetic variants in non-TSC1/2 identified in the LAM patients.
(XLSX)

S7 Table. Germline variants in non-TSC1/2 identified in the LAM patients.
(XLSX)

S8 Table. Somatic variants in non-TSC1/2 identified in the LAM patients.
(XLSX)

Acknowledgments
We sincerely appreciate the supports from the patients and their families. We thank all our team members for critical review of the manuscript.

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