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Mutation analysis of the *TSC1* and *TSC2* genes in Japanese patients with pulmonary lymphangioleiomyomatosis

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Abstract Pulmonary lymphangioleiomyomatosis (LAM) is a destructive lung disease characterized by a diffuse hamartomatous proliferation of smooth muscle cells (LAM cells) in the lungs. Pulmonary LAM can occur as an isolated form (sporadic LAM) or in association with tuberous sclerosis complex (TSC) (TSC-LAM), a genetic disorder with autosomal dominant inheritance with various expressivity resulting from mutations of either the *TSC1* or *TSC2* gene. We examined mutations of both *TSC* genes in 6 Japanese patients with TSC-LAM and 22 patients with sporadic LAM and identified six unique and novel mutations. *TSC2* germline mutations were detected in 2 (33.3%) of 6 patients with TSC-LAM and *TSC1* germline mutation in 1 (4.5%) of 22 sporadic LAM patients. In accordance with the tumor-suppressor model, loss of heterozygosity (LOH) was detected in LAM cells from 3 of 4 patients with TSC-LAM and from 4 of 8 patients with sporadic LAM. Furthermore, an identical LOH or two identical somatic mutations were demonstrated in LAM cells microdissected from several tissues, suggesting LAM cells can spread from one lesion to another. Our results from Japanese patients with LAM confirmed the current concept of pathogenesis of LAM: TSC-LAM has a germline mutation but sporadic LAM does not; sporadic LAM is a *TSC2* disease with two somatic mutations; and a variety of *TSC* mutations causes LAM. However, our study indicates that a fraction of sporadic LAM can be a *TSC1* disease; therefore, both *TSC* genes should be examined, even for patients with sporadic LAM.

Key words Pulmonary lymphangioleiomyomatosis (LAM) · Tuberous sclerosis complex (TSC) · Forme fruste · *TSC1* · *TSC2* · Germline mutation · Somatic mutation · Loss of heterozygosity (LOH)

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Introduction

Pulmonary lymphangioleiomyomatosis (LAM) is a rare disease that almost exclusively affects women of reproductive age and is characterized by hamartomatous proliferation of abnormal smooth muscle cells (LAM cells) along the alveolar wall, vessels and lymphatics in the lungs, and occasionally mediastinal or retroperitoneal lymph nodes (Corrin et al. 1975; Silverstein et al. 1974). Clinical manifestations of pulmonary LAM include recurrent spontaneous pneumothorax, progressive dyspnea on exertion, chylothorax, and hemoptysis (Corrin et al. 1975; Silverstein et al. 1974). The most common features of pulmonary function in patients with pulmonary LAM is the impairment of diffusing capacity and airflow obstruction (Chu et al. 1999; Corrin et al. 1975; Johnson and Tattersfield 1999; Silverstein et al. 1974; Urban et al. 1999). LAM has a diverse clinical course and some patients retain a good pulmonary function for a long period without antihormone therapy, although others show progressive deterioration leading to respiratory failure and cor pulmonale (Seyama et al. 2001). Although LAM has been commonly recognized as a sporadic, noninherited pulmonary disease (sporadic LAM), it has also been known to develop in about 1%–4% of patients with tuberous sclerosis complex (TSC) (TSC-LAM) (Castro et al. 1995; Gomez 1999). TSC is a hereditary disor-

der with an autosomal dominant trait, characterized by seizures; mental retardation; and hamartomas of the brain, retina, skin, heart, lungs, and kidneys (Gomez 1999; Short et al. 1995). TSC is a tumor-suppressor gene syndrome in which Knudson's two-hit theory (Knudson 1971) is applied for the development of TSC-associated hamartoma (Henske et al. 1996; Sepp et al. 1996) and patients with TSC have a germline mutation of either the *TSC1* or the *TSC2* gene (Jones et al. 1997, 1999; Wilson et al. 1996; Yamashita et al. 2000). The *TSC1* gene codes for a 130-kDa protein named hamartin, which is located on chromosome 9q34 (Van Slegtenhorst et al. 1997), and the *TSC2* gene codes for a 200-kDa protein named tuberin (Consortium 1993), located on chromosome 16p13.3.

Recent studies have indicated that angiomyolipomas and lymph nodes from patients with sporadic LAM have a *TSC2* loss of heterozygosity (LOH) but not a *TSC1* LOH (Smolarek et al. 1998). Patients with sporadic LAM were demonstrated not to carry the *TSC2* germline mutation (Astrinidis et al. 2000); inactivating mutations of the retained *TSC2* allele have been demonstrated in the affected tissues but not in unaffected tissues from patients with sporadic LAM (Carsillo et al. 2000). These findings clearly indicate that sporadic LAM is different from TSC-LAM with either the *TSC1* or *TSC2* germline and is caused by two somatic mutations of *TSC2* in individuals without TSC. In this study, we analyzed mutations of the *TSC* genes in 28 Japanese patients with LAM to confirm the recent advances in pathogenesis of LAM and we report six novel mutations.

Materials and methods

Study group

This study was approved by the Institutional Ethical Board of Juntendo University Hospital and the informed consent from patients was obtained prior to their enrollment in this study. A total of 28 female patients with a diagnosis of LAM, confirmed by pathological examination of the lung specimen, were enrolled. Six had a clinical diagnosis of TSC according to the recently revised diagnostic criteria (Gomez 1999) and were defined as having TSC-LAM, and the remaining 22 patients had sporadic LAM with no dermatological or neurological signs and symptoms characteristic of TSC.

Genomic DNA was isolated from peripheral blood leukocytes and/or from an Epstein-Barr virus (EBV)-transformed lymphoblastoid cell line (EBV-LCL) that was established from peripheral blood mononuclear cells. Total RNA was isolated from EBV-LCL using TRIzol (Life Technologies, Rockville, MD, USA), and guanidine isothiocyanate extraction with ultracentrifugation was used for isolating total RNA from frozen tissues such as the lung and lymph nodes. cDNA primed with oligo-(deoxythymidine)15–17 was synthesized from 5 μg of total RNA in 20 μl of reaction volume using a cDNA synthesis kit (Life Technologies).

Screening of mutations of the *TSC1* and *TSC2* genes

A dideoxy fingerprinting method (ddF) (Sarkar et al. 1992) was used for screening mutations of the *TSC* genes when RNA was available from EBV-LCL and tissue specimen. On the other hand, a single-strand conformational polymorphism (SSCP) method was used as described previously (Yamashita et al. 2000) when only genomic DNA was available for analysis. Reverse transcriptase-polymerase chain reaction (RT-PCR) (using cDNA as a template) of the *TSC* genes was performed using several pairs of primers designed to cover the entire coding region of each gene. The entire coding region of the *TSC1* gene was divided into two overlapping fragments and amplified separately by RT-PCR; a pair of P1 and P10 primers generated a DNA fragment (2030 bp) derived from the 5′ side of the *TSC1* gene and a pair of P9 and P17 generated DNA fragments (1911 bp) derived from the 3′ side of the *TSC1* gene (Table 1). Primers used for RT-PCR of the *TSC2* gene were adopted from the study reported by Wilson et al. (1996); three overlapping DNA fragments (1963 bp, 2357 bp, and 1411 bp) were generated. RT-PCR was performed using Expand Long Template PCR system (Roche Diagnostics, Tokyo, Japan) according to the manufacturer's instruction. The reaction mixture contained 1.0 μl of cDNA solution, 2.25 mM MgCl2, 500 μM deoxyribonucleoside triphosphate (dNTP), 300 nM primers, and 2.5 units of DNA polymerase mixture (Taq and Pwo polymerases) in 50 μl of reaction volume. The thermal cycler (Model 9600, PE Biosystems, Chiba, Japan) condition was 10 cycles of denaturation (93°C, 15 s), annealing (60°C, 30 s), and extension (68°C, 2 min), followed by 20 cycles of denaturation (93°C, 15 s), annealing (60°C, 30 s), and extension (68°C, 2 min and 20 s) with a 20-s increment of extension time for each cycle. RT-PCR products were loaded onto agarose gel electrophoresis and amplified DNA fragments of both *TSC1* and *TSC2* genes were cut from the gel and purified using Qiaex II DNA extraction kit (Qiagen, Tokyo, Japan). Purified DNA fragments were used to screen mutations of the *TSC1* or *TSC2* gene, respectively, by ddF; primers used for screening the *TSC1* gene by ddF are shown in Table 1 those for the *TSC2* gene were adopted from the study reported by Wilson et al. (1996). The ddC (2′,3′-dideoxycytidine 5′-triphosphate) sequence reaction was performed with the *ThermoSequenase* cycle sequencing kit (Amersham Pharmacia Biotech, Tokyo, Japan) according to the manufacturer's instruction. When an aberrant band was detected by ddF, nucleotide alteration was confirmed by sequencing (described following).

Sequencing of the *TSC* genes

To determine the sequence alteration, we amplified a part of the *TSC* genes by PCR using genomic DNA (Yamashita et al. 2000) or cDNA (described earlier); sources of genomic DNA were either peripheral blood leukocytes, EBV-LCL, or paraffin-embedded tissue specimens (described following). Amplified DNA fragments were purified from

Table 1. Oligonucleotide primer used in the analysis of the *TSC1* gene

Primer name	Sequence	Direction[a]	Location[b]
P1	5′-ACTGAAGTACCAGTTGTCGCTAG-3′	F	nt 114–136
P2	5′-ATCCTGACCACCTTGCAAGAGC-3′	F	nt 398–420
P3	5′-TGGCCGTCTGTCATCATGGTGC-3′	F	nt 695–716
P4	5′-GATGTTGTGATCGAGTGTGCCA-3′	F	nt 981–1002
P5	5′-TTACTCCACGTCTCGGCTGATG-3′	F	nt 1154–1175
P6	5′-GAAGATGGTGTTGTCTGTGTAG-3′	R	nt 1515–1536
P7	5′-ACTCTAAGTGATCTTCCAGGGT-3′	F	nt 1584–1605
P8	5′-GAAAGTTCTCTAGATATTGCAG-3′	R	nt 1660–1681
P9	5′-GCAAGCCTTTACTCCCATAG-3′	F	nt 1868–1887
P10	5′-GGCACACCATCTTCCTCTG-3′	R	nt 2125–2143
P11	5′-GACAGACTGATACAGCAGGGAG-3′	F	nt 2169–2190
P12	5′-CTAGATACAATCAGCTCCAGGA-3′	F	nt 2497–2518
P13	5′-GTCAACGAGCTCTATTTGGAAC-3′	F	nt 2793–2814
P14	5′-CAGGTGTTTGAATTGGAGATCT-3′	F	nt 3087–3108
P15	5′-GGCAGTAGTGGAAGCAGAGG-3′	F	nt 3303–3322
P16	5′-CATGACCAGTAGCCTTTCTGA-3′	F	nt 3545–3565
P17	5′-GCATTCACACCTCCTGTTCT-3′	R	nt 3759–3778

[a] F, forward primer; R, reverse primer
[b] The nucleotide number (nt) was adopted from the sequence data of the *TSC1* gene (GenBank, accession number AF013168); the A nucleotide of the first methionine (ATG) of the *TSC1* gene was assigned as nt 222

agarose gel as described earlier and followed by direct sequencing using [^{32}P]-labeled primers and *ThermoSequenase* cycle sequencing kit (Amersham Pharmacia Biotech). When cloning of the PCR product was required, a pTA cloning kit (Invitrogen, Carlsbad, CA, USA) was used according to the manufacturer's instruction.

Microdissection of smooth muscle cells from paraffin-embedded tissues

Formalin-fixed and paraffin-embedded tissue specimens were used to dissect proliferating smooth muscle cells (LAM cells) and control cells such as bronchial epithelial cells and lymphocytes, followed by DNA extraction and genetic analysis (Fujii et al. 1996). Multiple sections 8 μm in thickness were deparaffinized, stained with hematoxylin and eosin, visualized with an inverted microscope, and dissected using a 26-gauge needle. Microdissected smooth muscle cells were digested overnight in 15–30 μl of lysis buffer containing 50 mM Tris-HCl (pH 8.0), 1 mM ethylenediaminetetraacetate (EDTA), 0.5% NP-40, and 200 μg/ml proteinase K. The lysate was used directly in PCR for LOH analysis or preparing template DNA fragments for direct sequencing.

LOH analysis of microsatellite markers at chromosomes 9q34 and 16p13.3

Microsatellite markers were selected according to Smolarek et al. (1998): D9S149, D9S1198, and D9S1199 for chromosome 9q34, and D16S283, D16S291, D16S525, and Kg8 for chromosome 16p13.3. PCR was performed in 10 μl of reaction buffer (10 mM Tris-HCl at pH 8.3 at 25°C, 50 mM KCl, 0.1% Triton X-100) containing 1.0 μl of DNA solution, 1–2 mM MgCl2, 200 μM dNTP, 250 nM primers, 0.25 μl of [^{32}P]α-deoxycytidine triphosphate (97.5 MBq/mmol), and 0.5 units of *Taq* DNA polymerase (Toyobo, Osaka, Japan). Antibody against *Taq* DNA polymerase (CLONTECH Laboratories, Tokyo, Japan) was added according to the manufacturer's instruction to perform hot-start PCR to ensure proper amplification. The conditions for the thermal cycler were 10 cycles of denaturation (93°C, 30 s), annealing (60°C, 1 min), and extension (72°C, 1 min), followed by 25 cycles of denaturation (93°C, 30 s), annealing (58°C, 1 min), and extension (72°C, 1 min). After terminating the reaction by addition of 5 μl of stop solution (95% formamide, 5 mM EDTA at pH 8.0), PCR products were resolved by 6% polyacrylamide gel containing 8 M urea and 11% formamide followed by autoradiography. LOH was determined by visual inspection of the relative intensities of the bands in LAM cells in comparison to the pattern of normal cells obtained from the same tissue specimen. All analyses were repeated at least twice for confirmation of the results.

Results

Mutations of the *TSC* genes identified in patients with LAM

We identified six novel mutations, including three deletions, two insertions, and one nonsense mutation; three are germline mutations and the remainder are somatic mutations (Table 2). In two of six patients with TSC-LAM, germline mutations were identified. Both involved the *TSC2* gene: deletion of nt993G at exon 9 in patient LKM14 and deletion of nt4891A at exon 37 in patient LTK22. The mutation identified in patient LKM14 occurred at the splice donor site of the exon 9/intron 10 boundary. RT-PCR using total RNA isolated from EBV-LCL and subsequent cloning of PCR products revealed the generation of three different mRNA transcripts: transcripts skipping exon 9, those skip-

Table 2. Mutations of the *TSC* genes in patients with pulmonary lymphangioleiomyomatosis

Gene	Category of mutation	Location	Nucleotide alteration	Expected protein alteration	Reference[a]
TSC-LAM					
TSC1	Germline	ex 9	T1112G	Try297stop	Strizheva et al. 2001
TSC1	Germline	ex 10	C1222A	Ser334stop	Jones et al. 1999
TSC2	Germline	ex 9	G898A	Gly294Arg	Strizheva et al. 2001
TSC2	Germline	ex 16	C1849T	Arg611Trp	Jones et al. 1999
TSC2	Germline	ex 19	T2168G	Leu717Arg	Zhang et al. 1999
TSC2	Germline	ex 23	A2701G	Met895Val	Niida et al. 1999
TSC2	Germline	ex 38	C5042T	Pro1675Leu	Franz et al. 2001
TSC2	Germline	ex 39	C5144T	Pro1709Leu	Franz et al. 2001
TSC2	Germline	ex 41	C5401T	Arg1795Cys	Strizheva et al. 2001
TSC2	Germline	ex 7	G778T	Gln254stop	Strizheva et al. 2001
TSC2	Germline	ex 19	G2127A	Trp703stop	Niida et al. 2001
TSC2	Germline	ex 29	C3460T	Gln1148stop	Strizheva et al. 2001
TSC2	Germline	ex 30	C3773	Ser1252stop	Strizheva et al. 2001
TSC2	Germline	ex 9	465-bp deletion	Large deletion	Franz et al. 2001
TSC2	Germline	ex 9	Del921-940	Met301fs → aa330stop	Franz et al. 2001
TSC2	Germline	Many exons	Large deletion	Loss of tuberin	Jones et al. 1999
TSC2	Germline	ex 18	Del1978-79	Gly654fs → aa655stop	Franz et al. 2001
TSC2	Germline	ex 24	Del2832-2833TA	Thr938fs → aa958stop	Beauchamp et al. 1998
TSC2	Germline	ex 27	Del3232A	Ser1072fs → aa1081stop	Franz et al. 2001
TSC2	Germline	ex 37	Del4891A	Met1625fs → aa1671stop	this study (LTK22)
TSC2	Germline	ex 38	Del5069-5086+16	NA[b]	Franz et al. 2001
TSC2	Germline	ex 40	Del5256-5273	6 aa in-frame deletion	Strizheva et al. 2001
TSC2	Germline	ex 9	Del993G	Multiple splicing products	Maruyama et al. 2001 and this study (LKM14)
TSC2	Germline	int 18	G2116-1A	NA [b]	Franz et al. 2001
Sporadic LAM					
TSC1	Germline	ex 6	C716A	Cys165stop	This study (LNK8)
TSC2	Somatic	ex 16	G1850A	Arg611Gln	Carsillo et al. 2000
TSC2	Somatic	ex 10	G1114T	Glu366stop	Carsillo et al. 2000
TSC2	Somatic	ex 5	Del547-550CTTC	Leu177fs → aa180stop	Carsillo et al. 2000
TSC2	Somatic	ex 18	Del2079-2091	Ser687fs → aa693stop	Carsillo et al. 2000
TSC2	Somatic	ex 33	Del4267C	Arg1417fs → aa1475stop	This study (LMJ7)
TSC2	Somatic	ex 33	Ins4109T	Ser1364fs → aa1413stop	This study (LMJ7)
TSC2	Somatic	ex 40	Ins5196-5199TGCA	His1726fs → aa1729stop	This study (LIY26)

Mutations of the *TSC* genes identified in this study and collected from literature are summarized in this table. The nucleotide number of the *TSC1*gene is presented in the same manner as that of Table 1. The nucleotide number of the *TSC2* gene is presented by assigning the A nucleotide of the first methionine (ATG) of the *TSC2* gene as nucleotide number 19

aa, amino acid; bp, base pair; Del, deletion; ex, exon; fs, frame shift; Ins, insertion; int, intron; NA, not available

[a] The patient name was presented in the parenthesis

[b] Protein alteration could not be estimated because the precise nucleotide or splicing alteration was not presented in the original report (Franz et al. 2001)

ping exon 9 and a part of exon 10 because of generation of a cryptic splice acceptor site in exon 10, and those skipping exons 9 and 10 (data not shown). A deletion of nucleotide (nt)4891A at exon 37 of the *TSC2* gene in patient LTK22 is expected to result in a frameshift from the position of amino acid 1671 residue and premature termination of tuberin.

On the other hand, a germline mutation, Cys165(TGC) → TGA stop on exon 6 of the *TSC1* gene, was identified in only 1 (patient LNK8) of 22 patients with sporadic LAM (Table 2). The sequence of exon 6 of the *TSC1* genes from her parents and elder brother was normal (data not shown), suggesting a new mutation in the patient. However, the same nonsense mutation was detected using genomic DNA isolated not only from peripheral blood leukocytes but also from normal lung tissues (panel 1 in Fig. 1), normal renal tissues (data not shown), and EBV-transformed B lymphoblastoid cell line (data not shown), suggesting a *TSC1* germline mutation. Although this patient is considered genetically to be a TSC patient, the clinical phenotype was sporadic LAM because she had no clinical features characteristic of TSC. Repeated computed tomography scans of the brain failed to detect abnormalities such as cortical tubers, intracerebral calcifications, and subependymal nodules in the brain. Ophthalmologic examinations revealed no bilateral hamartomatous lesions in the optic fundi. These observations, together with the normal dermatological findings, led us to diagnose this patient as having sporadic LAM according to the clinical diagnostic criteria for TSC (Gomez 1999). Postmortem examination supported the clinical diagnosis of sporadic LAM and revealed generalized LAM lesions involving the lungs, the mediastinal lymph nodes, the kidneys, and the uterus. An examination of the brain was not allowed.

In five patients with sporadic LAM, frozen lung tissues (four patients) and lymph node (one patient) were available for mutation analysis of the *TSC* genes, and somatic mutations of the *TSC2* gene were identified in two patients (patients LIY26 and LMJ7) (Table 2). Insertion of TGCA at

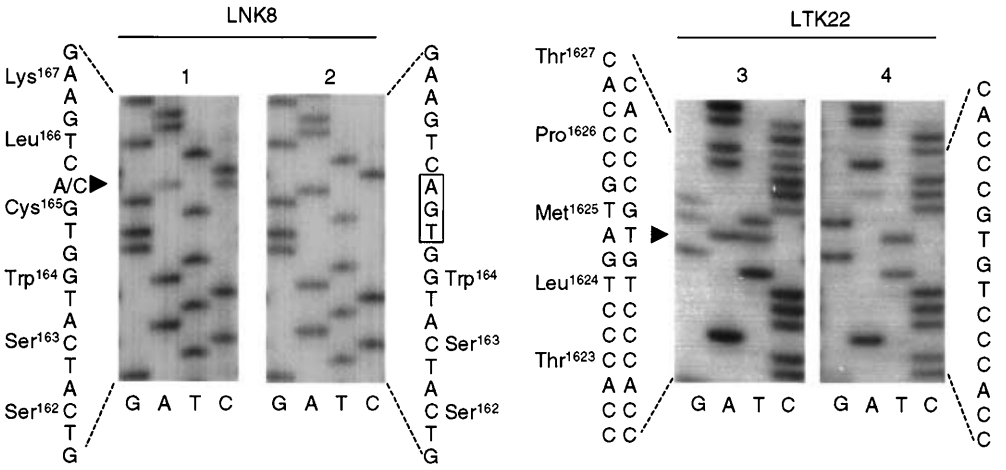

Fig. 1. Complete inactivation of the *TSC* genes in lymphangioleiomyomatosis (LAM) cells supports the tumor-suppressor model. Normal lung cells and LAM cells were microdissected from a formalin-fixed and paraffin-embedded lung specimen and the nucleotide sequences of the *TSC1* gene (patient LNK8) and the *TSC2* gene (patient LTK22) were compared. In patient LNK8, a sporadic LAM with a *TSC1* germline mutation (Cys165(TGC) → TGA), two bands (wild [*C*] and mutated [*A*] nucleotides) were observed at the same position in microdissected normal lung tissues (*panel 1*, indicated with an *arrowhead*) when sequences of the *TSC1* gene coding from Ser162 to Lys167 were analyzed. However, only a mutated sequence ladder (A nucleotide) is evident in LAM cells microdissected from both the left upper lobe (*panel 2*) and right kidney (data not shown), showing loss of the wild-type allele. In patient LTK22, a TSC-LAM with a *TSC2* germline mutation (nt4891A deletion), exon 37 of the *TSC2* gene of microdissected normal cells and LAM cells was sequenced. A part of the normal and mutated nucleotide sequences ranging from Thr1623 to Thr1627 is shown on the *left*. Compared with the superimposed bizarre ladders after the position indicated with the *arrowhead* (*panel 3*), demonstrating the mixture of normal and mutated ladders in the microdissected normal lung cells, only the mutated sequence ladder was obtained from the microdissected LAM cells (*panel 4*), clearly showing loss of the wild-type allele

exon 40 was identified in patient LIY26. On the other hand, two different mutations, insertion of a T nucleotide at nt4109 position and deletion of nt4267C at exon 33 of the *TSC2* gene, were identified in the lymph node but not in peripheral blood leukocytes from patient LMJ7, suggesting two somatic mutations of the *TSC2* gene. Cloning of PCR products of exon 33 revealed that two different mutations were on different alleles; no clone simultaneously carrying two mutations was identified. Because six lymph nodes were resected during surgery, LAM cells from the remaining five paraffin-embedded lymph nodes were microdissected to examine whether LAM cells in different lymph nodes carried the same *TSC2* somatic mutations as those identified in the frozen lymph node. Both an nt4109T insertion and an nt4267C deletion were detected as superimposing sequence ladders after the site of insertion or deletion in LAM cells was microdissected from each lymph node, whereas the sequence ladder in lymphocytes microdissected from one of the paraffin-embedded lymph nodes as a control showed a normal exon 33 sequence (Fig. 2).

LOH analysis of microdissected LAM cells

In 12 of 28 patients (4 patients with TSC-LAM and 8 patients with sporadic LAM), paraffin-embedded tissue specimens were available for microdissecting LAM cells and subsequent LOH analysis. As a whole, *TSC2* LOH was detected in six patients (three with TSC-LAM and three with sporadic LAM), whereas *TSC1* LOH was found in only one patient (patient LNK8, sporadic LAM with a *TSC1* germline mutation) (Table 3). *TSC1* or *TSC2* LOH at two different markers was detected in patient LNK8 (D9S149 and D9S1198) or in patient LSS6 (D16S291 and D16S525), respectively. The remaining five patients (patients LTT18, LKH2, LMJ7, LYS23, and LIY26) showed allelic loss neither at the *TSC1* nor at the *TSC2* locus. Representative results of LOH analysis of microdissected LAM cells are presented in Fig. 3 (patients LNK8, LTK22, and LJY20). There were two patients (LNK8 and LJY20) whose LAM cells were microdissected from different tissue specimens, and an identical LOH pattern was revealed in LAM cells among different specimens; both lungs, both kidneys, lymph nodes, and the uterus in patient LNK8 (Fig. 3), and angiomyolipoma and the lung in patient LJY20 (data not shown, but listed in Table 3).

Demonstration of two hits (inactivation of both alleles) in LAM cells

Two somatic mutations identified in LAM cells of patient LMJ7 (sporadic LAM) (Fig. 2) are very likely to indicate that LAM cells with loss of both *TSC2* alleles expanded clonally and spread into adjacent lymph nodes. Complete inactivation of the *TSC1* gene concordant with Knudson's tumor-suppressor model (Knudson 1971) was also observed in patient LNK8 who has a *TSC1* germline mutation and whose LAM cells have *TSC1* LOH at D9S149 and D9S1198 markers. When exon 6 of the *TSC1* gene was examined in

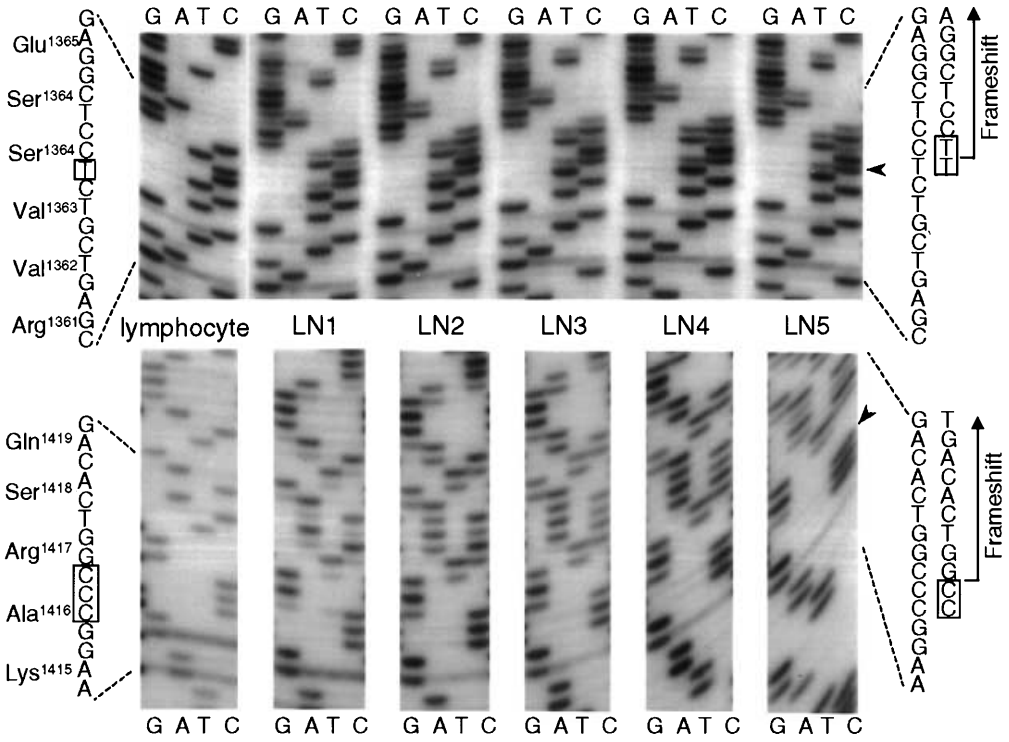

Fig. 2. Sequencing of exon 33 of the *TSC2* gene in patient LMJ7 using genomic DNA isolated from paraffin-embedded tissues. LAM cells were microdissected from formalin-fixed and paraffin-embedded retroperitoneal lymph nodes; five unrelated lymph nodes were arbitrarily numbered (LN1 to LN5). Lymphocytes were microdissected as a control from one lymph node. The sequence of exon 33 of the *TSC2* gene was examined using genomic DNA isolated from microdissected cells. The *upper panel* shows a part of the nucleotide sequence coding from Arg1361 to Glu1365 and the normal nucleotide sequence is shown on the *left*. Compared with the normal sequence obtained from control lymphocytes, two nucleotide sequences (shown on the *right*) are superimposed from the position of the C nucleotide of Ser1364(TCC) (indicated by the *arrowhead*) in LAM cells from LN1 to LN5; this is due to a T insertion between T and C nucleotides (TCC → TTCC) in one *TSC2* allele. In the *lower panel*, the nucleotide sequence from Lys1415 to Gln1419 is presented in the same manner as in the upper panel. Compared with the normal sequence obtained from the lymphocytes, two nucleotide sequences (shown on the *right*) are superimposed from the position of the C nucleotide of Arg1417(CGG) (indicated by the *arrowhead*) in LAM cells from LN1 to LN5 because of a C deletion (Ala1416Arg1417(GCCCGG) → GCCGG)

Table 3. LOH analysis in 12 patients with pulmonary lymphangioleiomyomatosis (LAM)

Patient	Paraffin-embedded tissue	Allelic loss[a]	
		TSC1 Markers	*TSC2* Markers
TSC-LAM			
LSS6	AML	None	D16S291, D16S525
LKM14	Lung	None	D16S291
LTT18	Lung	Not amplified	Not amplified
LTK22	Lung	None	D16S283
Sporadic LAM			
LKH2	Lung	None	None
LMJ7	LN	None	None[b]
LNK8	Lungs, kidneys, lymph nodes, uterus	D9S149, D9S1198	None
LMR11	Lung	None	D16S291
LYN17	Lung	None	D16S525
LJY20	Lung, AML	None	D16S283
LYS23	Lung	None	None
LIY26	Lung	None	None

Paraffin-embedded tissue blocks were available in 12 patients (4 patients with TSC-LAM and 8 patients with sporadic LAM) for microdissection of LAM cells followed by LOH analysis (see Material and methods)
AML, renal angiomyolipomas; LN, lymph nodes
[a] According to Smolarek et al. (1998), *D9S149*, *D9S1198*, and *D9S1199* were selected as microsatellite markers at chromosome 9q34 (*TSC1*), and *D16S283*, *D16S291*, *D16S525*, and *Kg8* were selected at chromosome 16p13.3 (*TSC2*). Microsatellite markers at which allelic loss was detected are shown in this table
[b] No allelic loss was detected but two somatic mutations in the *TSC2* gene were identified (see Table 2)

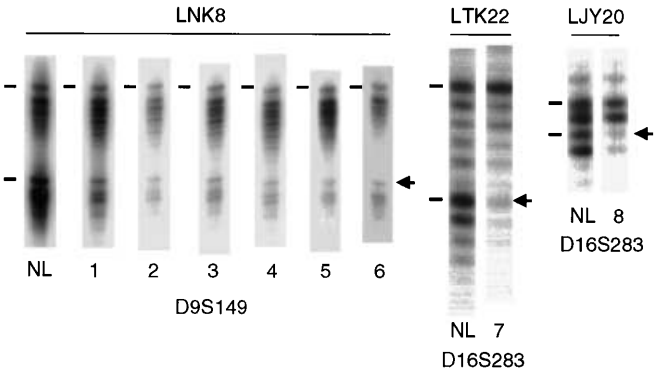

Fig. 3. Representative results of LOH analyses of microsatellite markers at chromosomes 9q34 and 16p13. The two alleles of each microsatellite markers are indicated with *lines* and the lost allele is indicated with an *arrow*. In patient LNK8, a sporadic LAM with a *TSC1* germline mutation, the lower allele of the marker D9S149 was lost in the LAM cells microdissected from the left upper lobe of the lung (*lane 1*), right lower lobe of the lung (*lane 2*), left kidney (*lane 3*), right kidney (*lane 4*), uterus (*lane 5*), and mediastinal lymph node (*lane 6*), whereas two alleles of the marker D9S149 were detected in the normal lung cells (NL). In patient LTK22, a TSC-associated LAM with a *TSC2* germline mutation, two alleles of the marker D16S283 were detected in the normal lung cells (*NL*), but the lower allele was lost in the LAM cells of the lung (*lane 7*). In patient LJY20, a sporadic LAM with no germline mutation, the lower allele of the marker D16S283 was lost in the LAM cells microdissected from the lung (*lane 8*) and renal angiomyolipoma (not shown) as compared with results of the normal lung cells (*NL*)

microdissected LAM cells, only the mutated sequence ladder was evident in LAM cells of the left lung (panel 2 in Fig. 1), showing a loss of the wild-type allele. The results were identical in LAM cells microdissected from the right lung, kidneys, mediastinal lymph nodes, and uterus (data not shown). On the other hand, two bands (wild and mutated nucleotides) migrated at the same position in the sequence ladder of normal lung tissues (panel 1 in Fig. 1). Identical findings are obtained in patient LTK22 who has TSC-associated LAM with a *TSC2* germline mutation (nt4891A deletion in exon 37) and whose LAM cells revealed LOH at D16S283. When exon 37 of the *TSC2* gene was analyzed, only the nt4891A-deleted sequence was observed because of loss of the wild-type sequence (panel 4 in Fig. 1) in LAM cells, whereas the sequence ladder superimposing the wild type and the deleted alleles in normal cells was observed (panel 3 in Fig. 1).

Discussion

We performed mutation analysis of the *TSC1* and *TSC2* genes in 28 Japanese patients with pulmonary LAM, consisting of 6 patients with TSC-LAM and 22 patients with sporadic LAM, and we report six novel mutations. Germline mutation was detected in two (33%) of six patients with TSC-LAM, both being deletions on the *TSC2* gene. One of two germline mutations detected (patient LKM14) was the splice site mutation first reported to be involved in LAM, which resulted in multiple splicing products of *TSC2* mRNA transcripts. Her clinical phenotype is characterized by multifocal micronodular pneumocyte hyperplasia and LAM of the lungs and has already been reported, with special emphasis on immunohistochemical findings (Maruyama et al. 2001). On the other hand, germline mutation was detected in one (4.5%) of 22 patients with sporadic LAM, whereas the majority of patients with sporadic LAM had neither of *TSC1* nor *TSC2* germline mutations. Our detection rate of germline mutations in patients with TSC-LAM appears to be almost identical to that of several studies on mutation analysis in patients with TSC (Au et al. 1998; Jones et al. 1997; Kwiatkowska et al. 1998; Van Bakel et al. 1997; Wilson et al. 1996; Yamashita et al. 2000) but lower than that of other studies (Cheadle et al. 2000; Dabora et al. 2001; Jones et al. 1999; Niida et al. 1999; Strizheva et al. 2001). The detection rate varies widely from 37% to 83%, depending on the screening method used. A recent study (Dabora et al. 2001) using a variety of screening methods, including heterpoduplex analysis, denaturing high-performance liquid chromatography, and quantitative PCR reported the highest detection rate (83%). The low detection rate in our study is likely due to the fact that the size of the TSC-LAM group is small or the fact that we only used SSCP and ddF among a variety of screening methods available. Thus we might have failed to detect *TSC* germline mutations in the remaining four TSC-LAM patients.

Pathogenesis and development of sporadic LAM has been established by a series of studies by Henske's group (Astrinidis et al. 2000; Carsillo et al. 2000; Smolarek et al. 1998). First, sporadic LAM is a *TSC2* disease; TSC-LAM is caused much less frequently by *TSC1* mutations than by *TSC2* mutations (Strizheva et al. 2001). Second, patients with sporadic LAM have no *TSC2* germline mutation (Astrinidis et al. 2000), whereas patients with TSC-LAM have a germline mutation (Strizheva et al. 2001). Lastly, sporadic LAM is caused by two successive somatic mutations of the *TSC2* gene, and LAM cells with complete inactivation of *TSC2* function can spread to from one lesion to another (Carsillo et al. 2000). Our study on Japanese LAM patients clearly support their observations: two of six patients with TSC-LAM had *TSC2* germline mutations; 21 of 22 patients with sporadic LAM had neither a *TSC1* nor a *TSC2* germline mutation; LAM cells showed LOH in three of four patients with TSC-LAM who were examined and in four of eight patients with sporadic LAM who were tested; complete loss of *TSC1* or *TSC2* function was demonstrated in LAM cells of one patient with sporadic LAM and in one patient with TSC-LAM, respectively; and finally, we detected that LAM cells microdissected from several tissues had identical LOH or two identical somatic mutations, suggesting LAM cells can spread from one lesion to another. In addition to confirming the established pathogenesis of LAM, our study suggests the importance of analyzing both *TSC1* and *TSC2* genes, not only in patients with TSC-LAM, but also in patients with sporadic LAM. Our identification of a phenotypically sporadic LAM that was genetically a forme fruste of TSC because of a *TSC1* germline mutation

underlines the importance of analyzing mutations of not only the *TSC2* but also of the *TSC1* gene. However, further studies are needed to determine the percentage of sporadic LAM patients who have a forme fruste of TSC-carrying *TSC* germline mutations. In addition, the fraction of patients with sporadic LAM from a *TSC1* disease remains unsolved; also unknown is whether there is a patient who has no germline mutation but develops sporadic LAM with two successive somatic mutations of the *TSC1* gene rather than a forme fruste of TSC, as revealed in patient LNK8.

There seems to be a variety of mutations causing LAM when the mutations reported in the literature are considered (Beauchamp et al. 1998; Carsillo et al. 2000; Franz et al. 2001; Jones et al. 1999; Maruyama et al. 2001, Niida et al. 1999, 2001; Strizheva et al. 2001; Zhang et al. 1999) and reviewed together with the mutations identified by our study (Table 2). There are 32 different mutations reported to cause LAM, 24 for TSC-LAM and 8 for sporadic LAM. No specific mutation has been reported to be frequently detected in patients with LAM except for Del5256-5273 on exon 40 of the *TSC2* gene found in two unrelated patients with TSC-LAM (Strizheva et al. 2001). As for TSC-LAM, two *TSC1* and four *TSC2* nonsense mutations, seven *TSC2* missense mutations, nine *TSC2* deletions, and two splice site mutations were reported. On the other hand, deletion and insertion are the most frequent type of mutation (five of eight mutations reported) in sporadic LAM followed by one each for the *TSC1* and *TSC2* nonsense mutations and one *TSC2* missense mutation. Recently, Strizheva et al. (2001) raised the possibility that mutations in exons 40 and 41 of the *TSC2* gene are associated with a higher incidence of LAM than are mutations elsewhere in the *TSC2* gene. All five *TSC2* mutations newly identified in our study are expected to lose the carboxy terminus of tuberin encoded by exons 40 and 41; in total, 22 (76%) of 29 *TSC2* mutations causing LAM are likely to fit into this category.

Why are *TSC2* mutations much more prevalent in LAM than are *TSC1* mutations? Since hamartin and tuberin associate together (Nellist et al. 1999; Plank et al. 1998; Van Slegtenhorst et al. 1998) and regulate the cell cycle (Miloloza et al. 2000; Soucek et al. 1997), it can be postulated that the development of LAM is due to the mutation of either the *TSC1* or the *TSC2* gene. It remains unclear why there is a higher predilection for a *TSC2* abnormality observed in LAM than in TSC in this and other studies (Carsillo et al. 2000; Smolarek et al. 1998), but it may be explained as follows. First, we and others might have missed enrolling sporadic LAM patients with *TSC1* mutations. It has recently been demonstrated that TSC patients with *TSC1* mutations are less severely affected in multiple clinical measures related to the brain, kidney, skin, and retina (Dabora et al. 2001; Jones et al. 1999). Because LAM is known to have a broad clinical spectrum, only the group of patients who manifest symptoms severe enough to seek medical attention because of *TSC2* abnormalities might enroll for genetic analysis. Second, the *TSC2* gene may be more susceptible to acquiring mutations because the *TSC2* gene is larger than the *TSC1* gene and spreads over about 44 kb of genomic DNA (The European Chromosome 16 Tuberous Sclerosis Consortium 1993). However, about half of the familial TSC cases showed linkage to the *TSC1* gene (9q34) and the remainder to the *TSC2* gene (16p13.3) (Povey et al. 1994), *TSC2* mutations account for about 70% of TSC cases (Cheadle et al. 2000) and are five times more common in sporadic TSC patients than are *TSC1* mutations. Dabora et al. (2001) reported that both germline and somatic mutations were less common in *TSC1* than in *TSC2* in a large cohort of TSC patients. The role of *TSC1* in the development of sporadic LAM, for which two successive somatic mutations are required, may decrease if *TSC1* is less prone to mutation than is *TSC2*. Lastly, the skewed mutational frequency may suggest that the hamartin and tuberin have distinct functions and tuberin commits more to the differentiation and proliferation of smooth muscle cells. Future studies are needed to compare the clinical severity of TSC-LAM associated with a *TSC1* germline mutation and TSC-LAM associated with a *TSC2* germline mutation, and to analyze mutations in sporadic LAM with a mild phenotype.

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