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Lymphangioleiomyomatosis (LAM): Molecular insights lead to targeted therapies

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Summary

LAM is a rare lung disease, found primarily in women of childbearing age, characterized by cystic lung destruction and abdominal tumors (e.g., renal angiomyolipoma, lymphangioleiomyoma). The disease results from proliferation of a neoplastic cell, termed the LAM cell, which has mutations in either of the *tuberous sclerosis complex (TSC) 1* or *TSC2* genes. Molecular phenotyping of LAM patients resulted in the identification of therapeutic targets for drug trials. Loss of *TSC* gene function leads to activation of mammalian target of rapamycin (mTOR), and thereby, effects on cell size and number. The involvement of mTOR in LAM pathogenesis is the basis for initiation of therapeutic trials of mTOR inhibitors (e.g., sirolimus). Occurrence of LAM essentially entirely in women is consistent with the hypothesis that anti-estrogen agents might prevent disease progression (e.g., gonadotropin-releasing hormone analogues). Levels of urinary matrix metalloproteinases (MMPs) were elevated in LAM patients, and MMPs were found in LAM lung nodules. In part because of these observations, effects of doxycycline, an anti-MMP, and anti-angiogenic agent, are under investigation. The metastatic properties of LAM cells offer additional potential for targets. Thus, insights into the molecular and biological properties of LAM cells and molecular phenotyping of patients with LAM have led to clinical trials of targeted therapies.
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Keywords

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Lymphangioleiomyomatosis (LAM) is a rare disease that affects primarily women of childbearing age1–5 with an incidence of approximately 2.6 per 1 million women.3 Organs involved in LAM include the lung, kidney (e.g., angiomyolipomas (AMLs)), and axial lymphatics (e.g., lymphangioleiomyomas, adenopathy).1–5 Patients with LAM present most frequently with dyspnea and pneumothorax1–5; other symptoms evident at presentation or during the course of the disease include chylothorax, hemoptysis, and ascites.[1–3,5] Patients usually first notice symptoms in their mid to late 30s,2–5 although diagnosis may be delayed for 5–6 years, in part due to similarities of the symptoms to those of more common lung diseases. Survival rate 10 years after diagnosis was approximately 85%6 or 90%.[7] The frequency of LAM is elevated among patients with tuberous sclerosis complex (TSC), an inherited disorder resulting from mutations in either the *TSC1* or *TSC2* genes. The clinical phenotype results from proliferation of the neoplastic LAM cell, which contains the TSC mutation.

Characteristics of LAM

Pulmonary dysfunction

The characteristic pulmonary radiologic (computed tomography (CT)) finding in patients with LAM is thin-walled cysts spread diffusely throughout the lung parenchyma, with no apical or basilar dominance.[1,5,6,8] Cystic changes, or the proliferation of LAM cells, are responsible for airflow obstruction and decreased lung diffusion capacity. FEV_1 (forced expiratory volume in one second) and DLco (diffusion capacity of the lungs for carbon monoxide) are reduced in approximately 60% of patients.[8] Evidence of air-trapping was observed on ventilation-perfusion scintigrams.[2,9]

Pathology

LAM results from the proliferation of abnormal smooth muscle-like cells (LAM cells), neoplastic cells that contain smooth muscle (α-smooth muscle actin (α-SMA)) and melanoma cell (gp100) antigens[10] as well as tuberous sclerosis complex (*TSC*) gene mutations.[11–13] Immunohistochemistry revealed two morphological types of LAM cells: epithelioid cells that react with HMB-45 (a monoclonal antibody that recognizes a splice variant of pmel-17 (gp100)) and spindle-shaped cells reactive with antibodies against proliferating cell nuclear antigen.[14] LAM cells proliferate in the vicinity of blood and lymphatic vessels, near bronchioles, and in walls of the cystic lesions.10 In the lung, LAM cell nodules in the lung are traversed by slit-like lymphatic channels, whereas in extrapulmonary lesions, LAM cells are seen in fascicles, which form plump rod-shaped bundles separated by lymphatic channels.15 Lung and lymphatic lesions and AMLs contain smooth muscle-like LAM cells. AMLs differ, however, from other LAM lesions in that they contain also underdeveloped vasculature and adipose tissue.[10,15–17] Hyperplastic type II pneumocytes are seen in LAM lung nodules.[18]

Pathogenesis

TSC is an autosomal dominant disorder, characterized by hamartomatous lesions, seizures, and mental retardation,[19] resulting from mutations in the *TSC1* or *TSC2* genes, which encode hamartin or tuberin, respectively.[20] Approximately one-third of women with TSC will present with pulmonary cystic lesions radiographically and histologically identical to

those in LAM.[21–23] *TSC2* mutations are much more frequent than those of *TSC1* in sporadic LAM patients.[11–13] In patients with LAM, who have received a lung transplant, *TSC* mutations identical to those present in the explanted lung were observed in the donor lung, consistent with metastatic properties of LAM cells.[24,25] Similarly consistent with a metastatic model of disease progression, LAM cells were also isolated from blood, urine, and chyle of LAM patients.[26]

LAM natural history study

More than 500 patients with LAM, primarily from the United States and Canada, but also from Europe and Southeast Asia, were enrolled in the LAM natural history protocol (NHLBI protocol 95-H-0186). In this longitudinal study, over 250 patients returned for five or more visits. Data on survival and disease progression from X-ray, biopsy, and/or physiological (e.g., pulmonary function tests) procedures were generated and collated.[5]

Predictors of time to death or transplantation

LAM histology scores

Severity of lung involvement in LAM was assessed in patients' lung biopsies using the LAM Histology Score (LHS). LHS is based on the extent of replacement of normal lung tissue by cystic lesions and LAM cell infiltrates.[27] The total percentage of tissue involvement by these two histologic patterns is graded as follows: LHS-1, <25%; LHS-2, 25% to 50%; and LHS-3, >50% of lung tissue involved. Using this grading method, significant differences in survival and time to transplantation for patients with LHS-1, -2, and -3 scores were observed (Fig. 1). The ten-year survival was found to be near 100% for LHS 1, 74.4% for LHS 2, and 52.3% for LHS 3.[27] These data confirmed prior observations showing that patients with more cystic disease have worse prognosis, and are more likely to have lower DLco and more exercise-induced hypoxemia than those with more muscular, solid lesions.[1] There was also a good correlation between DLco and FEV_1 and LHS[8] (Fig. 2).

Computed tomography

The severity of lung disease in LAM was graded semi-quantitatively by computed tomography,[9,28] according to the percentage of the abnormal lung in three equal pulmonary zones, represented by scans of three equal portions of images, using the scale of: 0, absent; 1, less than 30% abnormal; 2, 30%–60% abnormal; 3, more than 60% abnormal. Although good correlation of computed tomography with lung function tests was demonstrated, a predictive value of time to death or transplantation is not yet shown.[9,28]

Quantitative grading CT scans correlated well with measures of gas exchange and exercise performance.[29,30] Recent advances in computed tomography make possible the quantification of amount of lung parenchyma affected by lung cysts.[31] Percentage of cyst volume correlated with expiratory flow (FEV_1), residual volume and DLco.[30,31] The value of this radiologic procedure for prediction of survival or time to transplantation remains to be determined.

Pulmonary function tests

Pulmonary function testing is the simplest method of assessing severity of lung disease in LAM.[3,5,8,32] Abnormalities of pulmonary function were seen in >60% of patients. Both flow rates and lung diffusion can be normal, however, in as many as one third of patients. Although most patients have both airflow obstruction and impaired gas exchange, some may have normal flow or only mild airflow obstruction with a marked decrease in diffusion

capacity. The severity of lung disease in those patients should be graded by assessment of gas exchange such as DLco, arterial blood gases, or alveolar-arterial oxygen (A-a/O_2) gradient. These tests have limitations, however, as measurement of DLco is difficult to standardize and has a large intra-individual variability, whereas arterial blood gases at rest are very frequently normal.

No prospective study has established the levels of FEV_1 and DLco below which a LAM patient should be referred for transplantation. As in other pulmonary diseases, lung function criteria are only one component of the pre-transplant evaluation, and despite severe functional impairment, LAM patients can be relatively asymptomatic at rest. In general, however, by the time LAM patients undergo lung transplantation, they are on supplemental oxygen and have percent-predicted FEV_1 and/or DLco below 30% of predicted.[33] Still, even for patients in the last group, survival rate without lung transplantation is not possible to predict.

Exercise testing

Exercise testing in LAM may demonstrate abnormalities of gas exchange and/or ventilation and abnormal cardiovascular function.[33] Although lung function, especially DLco, can reasonably predict peak oxygen uptake (VO_2max), VO_2max is dependent on several additional factors, such as skeletal muscle function and cardiovascular fitness.[33,34] Exercise testing in LAM is important in that it elicits abnormalities not readily apparent during resting lung function, such as exercise-induced hypoxemia and pulmonary hypertension.[35] Because exercise-induced hypoxemia occurs in patients with mild impairment in lung function, cardiopulmonary exercise testing (CPET) or other types of exercise testing (e.g., six-minute walk test (6MWT)) may be useful measures of lung disease severity in LAM.[30,33]

An association between VO_2max and LHS scores was demonstrated, and patients with more severe LHS had significantly lower VO_2max.[33] Since LHS is a predictor of death and time to transplantation, VO_2max might also be a useful predictor of survival in patients with LAM.

Good correlation between a quantitative CT severity index and A-a/O_2 gradient, dead space/tidal volume ratio, and VO_2max was reported.[29] Further, we found that VO_2max, DLco, and FEV_1 correlated with the CT grade of disease severity[33] (Fig. 3). Moreover, good correlation between quantitative scores of cystic-aerial lesions obtained from CT scans and the 6MWT test have also been observed.[30] This is important because the 6MWT is a simple non-invasive test of proven value in assessing disease severity of patients with interstitial pulmonary fibrosis or pulmonary hypertension.[36]

Rate of functional decline

The rate at which lung function declines in LAM indicate how aggressive the disease is and, in an individual patient, has prognostic significance.[32] In one study involving 36 patients, the rate of FEV_1 decline was 118 ± 21 ml/year and for DLco was 0.905 ± 0.26 ml/min/mmHg/year.[37] Lazor et al.[38] reported an average decline in FEV_1 of 106 ± 35 ml/ year (3.4 ± 0.8 % predicted) in 31 patients followed for approximately five years. In a larger study of 275 patients followed for approximately four years,[32] the average yearly rates of decline in FEV_1 and DLco were 75 ± 9 ml (1.7 ± 0.4% predicted), and 0.69 ± 0.07 ml/min/mmHg (2.4 ± 0.4% predicted), respectively. Great variability in rates of disease progression is reflected in the large standard deviations. In some patients, LAM is a very aggressive disease with declines of FEV_1 or DLco from 5–10% predicted per year. Hayashida et al.[39] reported that in patients with FEV_1 above one liter and DLco above 40% predicted, those patients who

presented with dyspnea (n = 61), had a median decrease in FEV_1 of 285 ml (range −582.9 to −71.3 ml) per year and a median decrease in percent predicted DLco of 11.9% (range −20.7 to −3.2%). In patients who presented with pneumothorax (n = 74), loss of function was much slower with median annual decline in FEV_1 of 16.7 ml (range −157.1 to +46.8) or 1.6 % predicted (range −7.2 to +6.4%). Thus, LAM lung disease can sometimes progress quite slowly, taking decades to reach a level that interferes with activities of daily living or requires continuous oxygen therapy. In the absence of well-characterized markers of severity of this disease, FEV_1 and DLco (monitored frequently) are now the best methods to assess severity and progression of pulmonary disease in LAM.

Hormonal interventions

At present, no treatment is known to reverse effectively the functional abnormalities and prevent ongoing lung damage in LAM. Most systematically employed drug regimens involved anti-estrogen therapy,[5,32] based on the understanding of LAM as a disease predominantly of premenopausal women that may worsen during pregnancy[40–42] or following the administration of estrogens. 43–48 Consequently, most women with LAM were treated either with oophorectomy or long-term hormonal therapy, or both. These treatments became a standard of care, but no controlled studies evaluated their effectiveness. Most publications included only a handful of patients or were retrospective reviews. Taylor et al.[49] reported no improvement in 15 of 30 patients with LAM who had undergone bilateral oophorectomy; 11 patients worsened, and four were stable. The presence of estrogen or progesterone receptors in LAM lung lesions did not correlate with therapeutic response. Gonadotrophin-releasing hormone (GnRH) analogues were also found to be of benefit in the treatment of LAM,[50–52] although other studies were inconclusive.[53,54]

In a retrospective study, Johnson and Tattersfield[37] found that declines in FEV_1 in patients treated with progesterone were not significantly smaller than those in untreated patients, although their initial levels of FEV_1 and DLco were lower, and rates of decline over three years were not different. Decline in DLco was significantly less in 16 pre-menopausal patients treated with progesterone than in 13 pre-menopausal untreated patients, but the two groups were not matched. A decreased rate of functional decline was reported in five patients for whom pre- and post-treatment FEV_1 and DLco data were available. Another retrospective analysis of 275 patients[32] revealed that overall yearly rates of decline of FEV_1 and DLco were not significantly different in patients treated with oral or intramuscular progesterone and patients who received no progesterone after adjusting for differences in initial lung function, age, and disease duration.

In sum, although LAM is primarily a disease of pre-menopausal women, there is no clear evidence that treatments aimed at removing or antagonizing the effects of endogenous estrogens are effective.

Estradiol stimulated the growth of human angiomyolipoma *TSC2*$^{-/-}$ cells[32] and the pulmonary metastasis of *Tsc2*$^{-/-}$ Eker rat uterine leiomyoma-derived smooth muscle cells (ELT3 cells) in mice.[55] Enhancement by estrogens of the survival and growth of intravenously injected *Tsc2*$^{-/-}$ cells in mice55 was blocked by the MAPK/ERK kinase (MEK) inhibitor CI-1040.[55] Estrogen receptor activation in LAM cells increased matrix metalloproteinase (MMP)-2 activity promoting their invasiveness.56 Estrogens also accelerated growth of angiomyolipoma cells in a xenograft tumor model.57 All of these data suggest blockade of the MEK pathway as a new potential approach to the treatment of LAM. From these findings, anti-estrogen therapy may have a role in the treatment LAM, but precluding LAM cell metastasis could be crucial for its safety and/or effectiveness.

Association of modifier genes and biomarkers with disease severity and progression in LAM

Biomarkers linked to diverse LAM cell functions or disease characteristics could be useful as surrogates to follow disease progression and/or responses to therapy. Therefore, the discovery of modifier genes and biomarkers that are closely associated with clinical phenotypes of LAM is an important goal of LAM research.

Extracellular matrix proteins and pneumothoraces

Pneumothorax is one of the most frequent presentations of patients with LAM,5 with a rate of recurrence surpassing that in any other pulmonary disease.5,58 In a study of 227 patients with LAM,[59] the prevalence of pneumothorax was related to progression and severity of disease. Pulmonary function testing, CT grade, and cyst size of patients with LAM were compared to a history of pneumothoraces. Cyst size was graded by the average cyst diameter measured on high-resolution computed tomography (HRCT) using the following scale: size I < 0.5 cm; size II 0.5–1.0 cm; and size III > 1.0 cm. Patients with a history of pneumothorax were more likely to have larger cysts than those without. Multivariate analysis of a subgroup of LAM patients with mild disease and CT grade I revealed that FEV_1 declined more rapidly among those with a history of pneumothorax than those who did not, suggesting that early disease progression may be related to the occurrence of pneumothoraces.[59]

Collagens I and III are principal components of the alveolar wall scaffolding in the lung parenchyma.[60] Matrix metalloproteinase (MMP)-1 in LAM lung nodule is capable of degrading collagen and elastin.[61–63] It was hypothesized that the occurrence of pneumothoraces might be related to differences in genes involved in extracellular matrix formation or function.59 Patients with LAM were genotyped for polymorphisms in collagens I and III and MMP-1 to associate with susceptibility to LAM or the occurrence of pneumothorax. No correlation of polymorphisms with susceptibility to LAM was found, but patients with and without pneumothoraces differed in frequencies of polymorphisms in types I and III collagen and MMP-1 genes. An association of collagen III polymorphism with cyst size, although only a trend, was suggestive of a relationship between cyst size, type III collagen, and pneumothorax. Types I and III collagen and MMP-1 might be modifier genes affecting changes in the extracellular matrix that result in a greater susceptibility to pneumothorax and rate of decline in lung function.59

Lymphatic involvement in LAM

Lymphatic involvement in LAM includes lymphangioleiomyomas, adenopathy, and chylous pleural effusions. Adenopathy on CT scans appears as round and/or elliptical, well-circumscribed solid masses with replacement of normal tissue by smooth muscle (LAM) cells.[64] Avila et al.64 reported a 39% frequency of adenopathy in patients with LAM. Patients with abdominal adenopathy were more likely to have severe disease by CT grade.

Lymphangioleiomyomas, which occur in ca. 16%–21%[64,65] of patients with LAM, appear on CT scans as thin or thick-walled encapsulated lobulated masses containing LAM cells, which can be seen microscopically infiltrating the adipose tissue surrounding the fibrous capsule of the lesion.[15,64] Cysts contained fluid components. Lymphangioleiomyomas are thought to result from the proliferation of LAM cells, causing obstruction and subsequent dilatation of lymphatic vessels. Chylous ascites may occur from the rupture of distended lymphangioleiomyoma cysts. Lymphangioleiomyomas are usually found in conjunction with enlarged abdominal lymph nodes, pleural effusions, ascites, and dilatation of the thoracic duct.[64] In two radiologic studies, using sonography and/or CT scans,[65,66] 12 of 13 and 21 of 22 patients with LAM demonstrated diurnal size changes in

lymphangioleiomyomas. Observation of these changes on CT or sonography is helpful in the differential diagnosis of lymphangioleiomyomas and in the exclusion of lymphomas and other solid lymphatic masses that do not exhibit diurnal variation in size.

The identification of relatively specific lymphatic endothelial markers has facilitated analysis of the pathophysiologic role of lymphatic involvement in LAM. Examples are VEGFR-3 (FLT-4), a FMS-like tyrosine kinase 4 gene and a mediator of lymphangiogenesis[67]; VEGF-C and VEGF-D, both lymphatic growth factor ligands for VEGF-R368,69; podoplanin, a glomerular podocyte membrane mucoprotein recognized by antibody D2-40; and PROX-1, a transcription factor required for the differentiation of lymphatic endothelial cells.69,[70]

Immunohistochemical and immunocytochemical studies using specific lymphatic and vascular endothelial markers (e.g., CD31/PECAM, a platelet endothelial cell adhesion molecule and marker for vascular endothelial cells), as well as antibodies against HMB-45 and α-smooth muscle actin (LAM cell markers), demonstrated the extensive lymphatic involvement characteristic of LAM in the lungs and extrapulmonary tissue, including lymph nodes, uterus, and ovaries.71 LAM cells proliferated in the axial lymphatics, particularly the retroperitoneal area, including the diaphragm, thoracic duct, and left jugulosubclavian junction. 71,72 Anti-VEGFR-3 antibody reactivity of cells in the lining of the slit-like spaces within the LAM foci or nodules confirmed these structures as lymphatic tracts. Further, cells reactive with anti-VEGFR-3 antibodies, in mediastinal or retroperitoneal lymph nodes, appeared to segregate LAM cells into nodular or cluster-like arrangements. It was concluded that the observance of anti-VEGFR-3 reactive cells associated with LAM lung lesions indicated an active lymphangiogenic process linked to LAM. Consistent with these findings, microscopic observations revealed parts of LAM foci extending into the lymphatic lumen and clusters of LAM cells in lymphatic vessels.72 These LAM cell clusters (LCCs) were characterized as having an inner core of spindle-shaped cells, immunoreactive for anti-HMB-45, and an outer, single layer of flattened cells that react with anti-VEGFR-3 antibodies. LCCs were observed in chylous pleural and peritoneal effusions, and cystic lymphangioleiomyomas, as well as in the lung and in lymphatic channels in fat tissues surrounding lymph nodes containing LAM cells.[72] Based on these results, it was proposed that the shedding of LCCs by lymphangiogenesis-mediated fragmentation of LAM lesions, and their subsequent entry into the lymphatic circulation was a mechanism for the dissemination of LAM cells.

Immunoreactive VEGF-C and VEGF-D are both present in LAM cells.71,[73] Serum levels of VEGF-C and VEGF-D quantified by enzyme-linked immunosorbent assay (ELISA) in 44 patients with sporadic LAM were compared to serum levels in 24 healthy volunteers. Serum of VEGF-C in patients with LAM was significantly lower than that in healthy volunteers. [73] In two separate studies,73,74 serum VEGF-D levels were significantly higher in LAM patients than in controls (Fig. 4A). The higher levels of VEGF-D correlated with more severe disease (CT grade),[74] and worse FEV_1/FVC ratio, DLco (adjusted for lung volune),[73] and percent-predicted DLco,74 all consistent with a role for VEGF-D as a growth factor related to lymphangiogenesis in LAM.

When encountering a patient with pulmonary LAM in the absence of extrapulmonary manifestations (such as AMLs), distinguishing LAM from other cystic diseases can be problematical without performing a biopsy. Young et al.[75] measured serum levels of VEGF-D in 38 LAM patients, 24 healthy controls, and 27 patients with other pulmonary diseases (Langerhans-cell histiocytosis, lymphangiomatosis, and emphysema). Serum levels of VEGF-D in patients with LAM were significantly higher (as much as 30 times) than those in the other groups. Based on these results, serum VEGF-D might seem a potential biomarker

for LAM. In a study of a larger cohort of patients with LAM (LAM $n = 111$; healthy volunteers $n = 40$),[74] however, statistic significance of the difference in serum VEGF-D levels of LAM versus controls was not maintained when the LAM population was divided into 2 groups based on lymphatic involvement (Fig. 4B). Serum VEGF-D remained significantly different only when normals were compared to LAM patients with lymphatic involvement, not when compared to patients with only pulmonary disease, regardless of the presence or absence of AMLs. Consequently, low serum VEGF-D levels may reflect confinement of the disease to the lung. Results of the lymphatic growth factor studies indicate that lymphatic involvement may occur with disease progression and that levels of VEGF-D likely reflect lymphatic involvement in LAM, but may not be useful for the diagnosis of LAM in patients who have only cystic disease. Given these data, we suggest that clinical therapies targeted at VEGF-D and/or VEGFR-3 may slow lymphatic proliferation in LAM and possibly disease progression. Potential therapies include a mouse monoclonal antibody (VD1) that competes with mature VEGF-D for binding to VEGFR-2 and VEGFR-3, thereby inhibiting its function.76 In a mouse tumor model, VD1 inhibited VEGF-D-induced formation of lymphatics and tumor growth. Development of a human version of this antibody is in progress, 69,77 and other drugs targeting the VEGFR-3 pathway (e.g., AZD2171, sorafenib, sunitinib) are in clinical trials.78

Metastasis and LAM

LAM is characterized by the proliferation of smooth muscle-like cells that are disseminated in blood, urine, and chylous effusions[26] and may be able to form lung metastases.[79] In other types of cancer, the multistep metastatic process or “invasion-metastatic cascade” starts with a primary neoplasm that undergoes progressive growth followed by vascularization, invasion, detachment, embolization, cell survival in the circulation, arrest, extravasation, evasion of host defenses and growth at metastatic sites.[80] Since all of these steps are critical, blocking any of them could block the metastatic process, which involves “crosstalk” between the metastatic cell and its microenvironment.

The CD44 gene in chromosome 11 encodes 20 exons that undergo complex splicing to yield at least 10 different splice variants.[81] The multiple interactions of CD44 proteins suggest its participation in cell binding to extracellular matrix, endothelial cells, and other cells in the neoplasm microenvironment.[82] The metabolism and processing of CD44 presents interesting options for targeting. Splicing factors Sam68 and SRm160, which are activated by the Ras/MAPK pathway, regulate expression of CD44 forms[83–85] and Ras activation appears to be regulated by CD44v6.[83] Further investigation into the RAS/MAPK pathway downstream effects on CD44 splicing factors may elucidate therapeutic targets for controlling metastasis of LAM cells.

Osteopontin, an acidic 32.5-kDa phosphocytokine that is upregulated by TGF-β,[86] EGF,[87] and progesterone,[88] not only binds CD44,[89] but also regulates its splicing.[90] Ostepontin has been correlated with several metastatic processes and CD44v6 expression. The finding of elevated osteopontin levels in serum of patients with pulmonary LAM,[91] suggested that blocking its production could interfere with disease progression. Amounts of cleaved CD44 forms have been correlated with disease progression, and metalloproteases[92] and ADAM10[93] cleave CD44 to modulate cell attachment to extracellular matrix. Modification of CD44 enzymatic cleavage as well as of its splicing and turnover could involve several potential therapeutic targets.

Expression of the CD44v6, a splice variant of CD44, was detected in squamous cell carcinoma (head and neck, esophagus, lung, skin, cervix, vulva), adenocarcinoma (breast, Barrett’s esophagus, lung, gastric, pancreas, colon/rectum endometrium, prostate) and other tumors (thyroid carcinoma, small cell lung cancer, renal cell carcinoma, urinary bladder,

ovarian cancer and basal carcinoma).82 The presence of CD44v6 in so many cancers spurred researchers to target the molecule94 using antisense technologies, antibodies (U36, chU36, BIWA 4),95,96 and peptides (patent JP 2006083122). The anti-CD44 antibody H90 was notably successful in eradicating acute myeloid leukemia.97 As most approaches were directed at the primary tumor, very little was done to target the actual metastatic cells. CD44v6 is found in LAM lesions that have *TSC2* loss of heterozygosity, as well as in cultured cells grown from LAM lungs, similar to observations in other metastatic cells.[91] Presence of the CD44v6 splice variant on circulating LAM cells means that interference with its specific interactions could prevent anchoring of the cell to a metastatic site.

Cancer cells, regulated by the interaction of chemokines and their receptors, localize to sites of metastasis. Production of chemoattractants mainly at the metastatic site was shown for breast cancer and melanoma cells.[98] The metastatic cells display a defined group of receptors that are activated by chemokines produced by the normal stromal cells. LAM cells with identical mutations have been found in both lung and kidney of the same individual,[11] consistent with metastasis, but the sites of origin were not determined. We have observed that LAM cells within the lung lesions reacted with antibodies raised against CCR2, CXCR4, CCR7, or CCR1, and cells that were selectively mobilized by CCL2 / MCP1 showed loss of heterozygosity at the *TSC2* locus.[99] In addition, tuberin appeared to alter CCL2 expression.[100] These findings have led to the proposal of a positive feedback of selective chemoattraction. Multiple drugs that target CCR2[101] are known. We speculate that blocking the CCL2/CCR2 interaction via drug therapy might prevent metastasis of LAM cells and, therefore, disease progression. Multiple approaches, including the use of truncated MCP-1, anti-MCP-1 and aptamers, have been used to inhibit the function of CCL2.[102,103]

Matrix metalloproteinases

Matrix metalloproteinases (MMPs) are zinc-dependent endopeptidases critical in physiological embryogenesis and tissue remodeling. Dissociation of the cysteine-zinc complex produces an active zymogen capable of degrading extracellular matrix substrates. 104 This activation is inhibited by endogenous tissue inhibitors of metalloproteinases (TIMPs).104 MMP-9 and MMP-2 are able to degrade a variety of matrix substrates including gelatin, collagen I, collagen IV, laminin, and elastin.104 MMP activity is believed to facilitate tumor cell invasion by disrupting basement membranes.105 Concentrations of MMP-9 (in serum/plasma, and protein activity) have consistently correlated with the presence of cancer106,107 or metastasis108,109 and disease progression. [110,111] Levels of MMP-2 and MMP-9 activity in urine were predictive of disease status in a variety of cancers.[112] Degradation of elastic fibers in areas of smooth muscle proliferation was found in light and electron microscopic immunohistochemical observations of LAM lung biopsy specimens.[61] In agreement, immunohistochemical analysis of LAM tissue from lung biopsies indicated greater reactivity of MMP-2 and MMP-9, but not of TIMP-1 and TIMP-2, in LAM cells than in normal lung tissue.[62] MMP-2 and type IV collagen colocalized in some LAM cells.[62] In another study63 MT1-MMP, an activator of MMP-1, was associated with proliferating LAM cells, all suggesting from these studies a role for metalloproteinases in the cystic lung destruction of LAM. Because doxycycline is a MMP inhibitor that affects growth and migration of neoplastic cells, angiogenesis, lymphangiogenesis, and smooth muscle cell growth,113,114 MMP-9 and MMP-2 were proposed as biomarkers to measure disease progression during doxycycline therapy in a patient with LAM.[115] Levels of MMP-2, MMP-9, and MMP-9 complexed with neutrophil gelatinase-associated lipocalin (NGAL), which inhibits MMP-9 degradation,[116] were elevated before initiation of doxycycline treatment. After seven months of treatment with doxycycline, patient lung function had improved and MMP levels had decreased.115

Cyclin-dependent kinase 2 (CDK2) inhibitors

CDK2 inhibitors are potentially another therapeutic option for LAM.[117] Tuberin is a negative regulator of cell cycle progression that shortens the G1 phase of the cell cycle by binding p27KIP1, a cyclin-dependent kinase inhibitor, preventing its degradation and leading to inhibition of the cell cycle. In the absence of tuberin, p27 is mislocalized in the cytoplasm, allowing progression of the cell cycle.[118–121] Tuberin prevented degradation of p27, a major regulator of cell cycle progression, increasing the amount of p27 bound to CDK2. CDK2 inhibitors such as roscovitine, a new potential anti-cancer therapy,[122] may have a role in the therapy of LAM.

Regulation of TSC1- and TSC2-dependent pathways in LAM cells by mTOR

In LAM cells, mutations in the *TSC2* or *TSC1* genes resulted in defective regulation of a key protein kinase, mTOR. Further investigations of *TSC1*- and *TSC2*-dependent signal transduction pathways have led to promising pharmacological targets. mTOR (mammalian target of rapamycin) is an intracellular serine/threonine kinase that is responsible for mediating signals of energy availability and growth factor stimulation to regulate cell growth and metabolism (Fig. 5). Two different complexes contain mTOR: mTORC1, comprising mTOR, mLST8 (mammalian lethal with SEC13 protein 8), and raptor (regulatory associated protein of TOR); and mTORC2, containing mTOR, mLST8, rictor (rapamycin-insensitive companion of TOR), mSIN1 (mammalian stress-activated-protein-kinase-interacting protein 1), and PRR5 (proline-rich protein 5).[123–125] The substrate specificity of mTORC1 is determined by raptor.126 mTORC1 is acutely sensitive to rapamycin, whereas mTORC2 is sensitive only to prolonged rapamycin treatment.127,[128]

TSC1 and TSC2 form a cytoplasmic complex[129–131] that acts upstream of mTOR to integrate growth factor, energy, and stress signals and thereby regulate protein synthesis and cell growth and proliferation. *TSC1*, on chromosome 9q34,[132] encodes the ca. 130-kDa protein hamartin, which regulates levels of Rho-GTP. It interacts with proteins of the ezrin-radixin-moezin family and with tuberin, the TSC2 protein,[130,133–135] encoded by *TSC2* on chromosome 16p13.3.[136] The 198-kDa tuberin protein molecule contains a region with significant similarity to GTPase-activating proteins and a region that binds hamartin, [130,133,137–139] which stabilizes it and prevents its ubiquitin-mediated degradation.[140]

The TSC1/2 complex is a negative regulator of mTORC1. TSC2 is a GAP (GTPase-activating protein) for the GTPase Rheb (Ras homolog enriched in brain), that accelerates its Rheb conversion from the active Rheb-GTP to an inactive Rheb-GDP.137–139 Rheb-GTP activates mTORC1, resulting in phosphorylation of substrates such as ribosomal S6 kinases, e.g., S6K1, and eukaryotic initiation factor 4E-binding proteins, e.g., 4E-BP1.141 These phosphorylations lead to S6 kinase activation and 4E-BP inhibition, resulting in enhanced translation of 5′ TOP (terminal oligopyrimidine tract)-containing RNAs and cap-dependent translation. In response to growth factors, Akt phosphorylates and inactivates TSC2,142,143 which relieves the TSC1/2 inhibition of Rheb, allowing mTORC1 activation and promoting translation. 144–146 Under poor growth conditions, FKBP38 binds mTORC1 and inhibits its activity.[147] Rheb-GTP enables activation of mTORC1 by preventing the interaction of FKBP38 and mTOR.[147] Phosphorylation of TSC2 by Akt may lead to its binding of 14-3-3 and its resulting inactivation.[148]

Growth factors cannot stimulate mTORC1 without a supply of amino acids or cellular energy (in the form of ATP) sufficient to fuel translation. Under conditions of energy stress as detected by increased intracellular levels of AMP, activated AMPK (AMP-dependent protein kinase) phosphorylates TSC2.149 This phosphorylation activates TSC2149 and thereby inhibits mTORC1. Hypoxia also causes AMPK activation, as oxygen is required for

aerobic mitochondrial production of ATP by oxidative phosphorylation. Hypoxia stimulates HIFα (hypoxia-inducible factor α), which increases transcription of REDD1 that binds 14-3-3 proteins and can facilitate dissociation of Akt-induced TSC2/14-3-3 complexes, thereby activating TSC2 and inhibiting mTORC1.148

Ample supply of amino acids is needed also to permit localization of mTORC1 correctly for activation by Rheb. Amino acids stimulate GTP binding by Rag (Ras-related small GTP-binding protein)-GTPase heterodimers, which are able to bind raptor, and result in localization of mTORC1 in compartments where Rheb is able to activate mTORC1.[150] This translocation of mTORC1 stimulated by amino acids allows the mTORC1 activation and mRNA translation only when substrate sufficient for translation is present.

The TSC1/2 complex is a positive regulator of mTORC2, resulting in phosphorylation of Akt.[151,152] TSC1/2 physically interacts with mTORC2, and levels of TSC2 expression are correlated with increases in Akt phosphorylation.[151] Loss of TSC1/2 in disease leads to an inability to activate Akt, through both the inactivation of mTORC2 and through a negative feedback loop created by activated mTORC1, which blocks growth factor-stimulated phosphorylation of Akt.[123,151] As Akt is an important kinase in tumor progression, an inability to activate Akt may result in a more “benign” disease such as TSC, where activation of mTORC1 may initially result in tumor formation, but the negative feedback loop on Akt phosphorylation and inactivation of mTORC2 may limit disease progression.[151]

Rapamycin was approved in 1997 for immunosuppression in kidney transplant patients, and in 2003 for use in cardiovascular stents to prevent smooth muscle cell proliferation. 153 Rapamycin and FKBP-12 (FK506 binding protein-12) forms a complex that binds and inhibits mTORC1.[154] mTORC1 responds acutely to rapamycin, whereas prolonged rapamycin treatment can inhibit mTORC2. The differences in sensitivity have been proposed to be due to phospholipase D (PLD) action and the phosphatidic acid (PA) product.155›156 PLD activity generating PA from phosphatidylcholine hydrolysis was elevated in human cancer cell lines.157¯159 PA, which is required for the formation/stabilization of mTOR complexes, binds the FKBP12-rapamycin domain, competing with rapamycin for FKBP12 binding.[160] It was proposed that the apparent differences in sensitivity of the two mTOR complexes reflect a difference in the affinities for PA, such that mTORC2/PA is a more stable complex than mTORC1/PA, rendering mTORC1 more sensitive to rapamycin and requiring a more prolonged treatment with rapamycin to inhibit mTORC2.[155,156]

Effects of rapamycin or sirolimus have been investigated on tumors or renal cysts in mouse and rat models of TSC. CCI-779 (temsirolimus, a rapamycin analog) reduced the number of cystadenomas per kidney in *Tsc2*$^{+/-}$ mice.[161] Rapamycin treatment of the Eker rat, which carries a germline mutation of *Tsc2* that is functionally null, diminished tumors of the kidney and suppressed phosphorylation of S6 and 4E-BP1.[162] There were, however, rare persistent renal tumors with hyperphosphorylated S6 proteins after rapamycin treatment, consistent with resistance to rapamycin.

Several ongoing clinical trials are assessing the effect of rapamycin or its analogs on AML size in TSC and/or LAM patients. Some of the trials evaluate also effects on pulmonary function in LAM patients. A 24-month nonrandomized study[163] of TSC and/or LAM patients examined the effects of 12 months of sirolimus on AML volume and pulmonary function (in the LAM patients), and then followed the patients another 12 months without drug. AML volume was smaller at 12 months (53% of baseline), whereas, without sirolimus, the AMLs were 86% larger (86% at 24 months). Some improvement in airflow and gas trapping was found in LAM patients although there was no effect on DL_{CO}. Whereas the

effects of sirolimus tended not to be permanent, improvements in spirometry of some LAM patients persisted after treatment stopped. Information about clinical trials of rapamycin and its analogs on disease progression in TSC and LAM patients can be found at www.clinicaltrials.gov.

Conclusion

The discovery of an effective treatment for LAM is thus far elusive. Data from a longitudinal natural history study defined predictors of time to transplantation or death and identified extra-pulmonary manifestations of LAM. Molecular insights and the discovery of possible biomarkers in LAM have led to potential therapeutic options and means to monitor effects of therapy. Detection of estrogen receptors in LAM lesions and differences in post- and pre-menopausal disease progression suggest that hormonal intervention may offer benefits, and further study is warranted to establish the effectiveness of specific interventions. The isolation of LAM cells from blood and other body fluids offers a possible non-invasive diagnostic tool. VEGF-D may be a marker for lymphatic involvement in LAM and anti-VEGF-D therapies may slow lymphatic proliferation in LAM. Elevation of MMP-2/MMP-9 levels suggests doxycycline as a useful treatment, and CD44v6 may represent an effective target for cytotoxic agents. Elucidation of the effects of *TSC* gene mutations on the mTOR pathway has led to a clinical trial using sirolimus.

The use of pharmacological agents against these molecular targets has advantages as well as raising questions. Side effects of anti-estrogen therapy, such as loss of bone mineral density, may preclude the use in most patients of those presently available. MMP inhibitors, that are not selective for a particular enzyme, may produce off-target effects. The effectiveness of any treatment may depend on disease stage or many other physiological variables for each individual. All of these factors should be considered in designing treatment studies.

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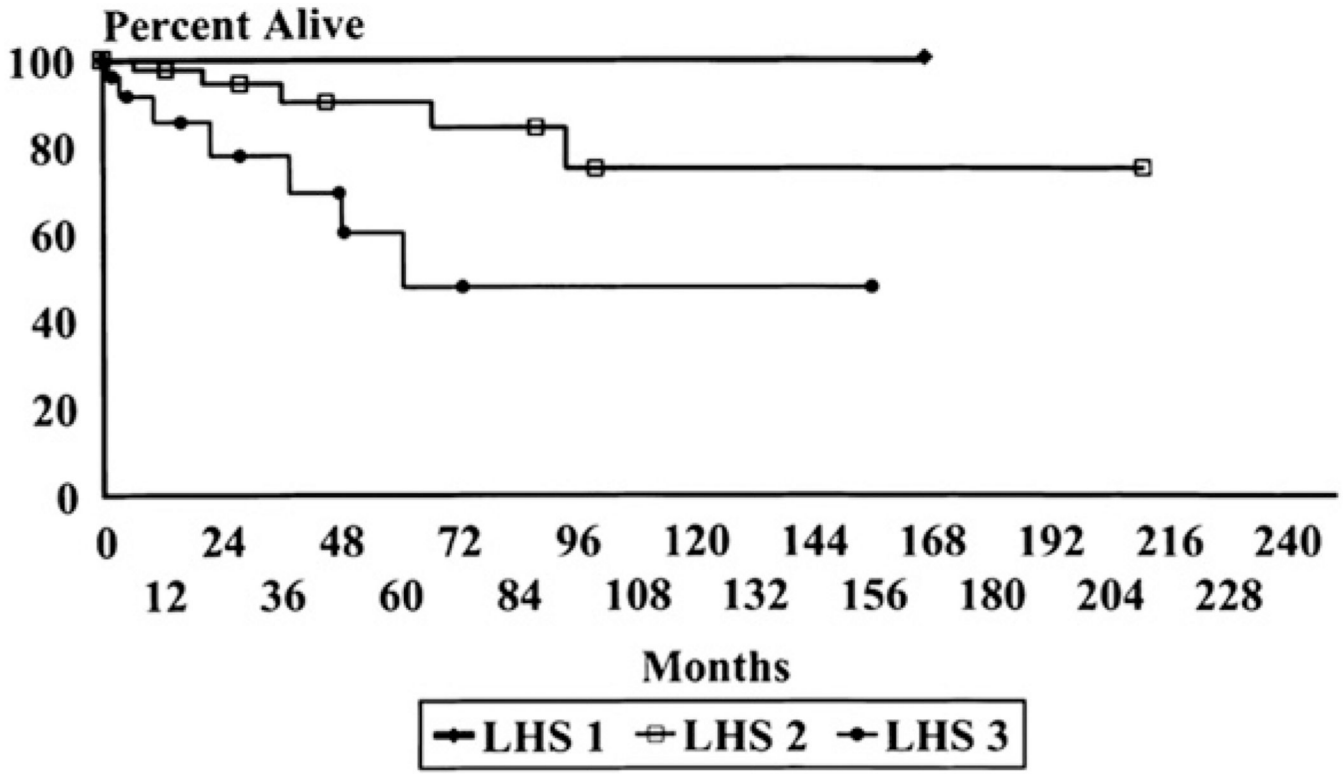

Figure 1.
Kaplan–Meier survival curves of patients with pulmonary lymphangioleiomyomatosis staged according to the lymphangioleiomyomatosis histologic score (LHS). Patients with LHS-1 have nearly 100% survival. Patients with LHS-3 have the worst survival, and those with LHS-2 have an intermediate survival ($p < 0.002$) (From reference 27).

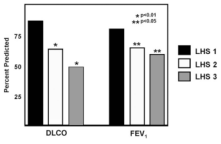

Figure 2.

Relationship between LHS and lung function at the time of biopsy. Patients with an LHS of 2 (white bars) or 3 (gray bars) have significantly lower DL_{CO} than those with an LHS of 1 (black bars). Patients with an LHS of 2 (white bars) or 3 (gray bars) also have lower FEV_1 than patients with an LHS of 1 (From reference 8).

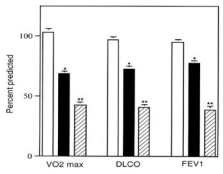

Figure 3.
Relationship between VO_2max, FEV_1, and DL_{CO} and CT grade of disease severity. Grade I, white bars; grade II, black bars; grade III, cross-hatched bars (From reference 33).

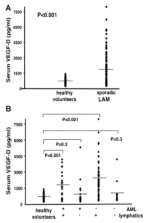

Figure 4.

Serum Levels of VEGF-D in Lymphangioleiomyomatosis. In Panel A, serum VEGF-D levels in all patients with sporadic lymphangioleiomyomatosis (LAM) (*n* = 111) were compared to those of healthy volunteers (*n* = 40). Panel B shows patient samples compared on the basis of thoracic or abdominal lymphatic involvement (presence (*n* = 77) or absence (*n* = 34) of lymphangioleiomyomas and/or adenopathy) and the presence (*n* = 40) or absence (*n* = 71) of renal angiomyolipomas (AMLs). All groups were compared to healthy volunteers (*n* = 40). (+) = presence of, (−) = absence of. Each ♦ represents serum measurement of VEGF-D from one patient or healthy volunteer. Lines represent mean values (From reference 74).

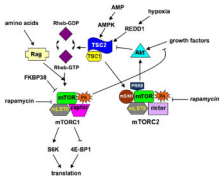

Figure 5.

mTOR dependent pathways. Two different complexes contain mTOR: mTORC1, which is acutely sensitive to rapamycin, and mTORC2, which is inhibited by rapamycin (in italics) after prolonged exposure. Activation of mTORC1 leads to protein translation, and thereby cell growth and proliferation, while activation of mTORC2 results in the phosphorylation of Akt. Growth factors, hypoxia, and energy stress affect the status of the TSC1/2 complex, leading to inactivation of the tumor suppressor complex in the presence of growth factors and activation in the presence of stress. TSC1/2 inhibits mTORC1 through its Rheb-GAP activity: Rheb-GTP is necessary for the activation of mTORC1, preventing the interaction of MTOR with its endogenous inhibitor FKBP38. The presence of amino acids stimulates the localization of mTORC1 in Rheb-containing compartments through the actions of Rag-GTPases. mTORC1 is also involved in a negative feedback loop, which blocks growth factor-stimulated phosphorylation of Akt. TSC1/2 activates mTORC2, which then phosphorylates Akt.