Immunohistochemical Evaluation of Pulmonary Lymphangioleiomyomatosis

IRMINA GRZEGOREK[1], DIDO LENZE[2], MARIUSZ CHABOWSKI[3,4], DARIUSZ JANCZAK[3,4], MAŁGORZATA SZOLKOWSKA[5], RENATA LANGFORT[5], ANDRZEJ SZUBA[6,7] and PIOTR DZIEGIEL[1]

[1]*Department of Histology and Embryology, Wroclaw Medical University, Wroclaw, Poland;*
[2]*Institute of Pathology, Charité Medical University, Berlin, Germany;*
Departments of [3]Surgery and [6]Internal Medicine, Fourth Military Hospital, Wroclaw, Poland;
Departments of [4]Clinical Proceedings and [7]Angiology, Faculty of Health Science, Wroclaw Medical University, Wroclaw, Poland;
[5]*Department of Pathology, National Tuberculosis and Lung Diseases Research Institute, Warsaw, Poland*

Abstract. *Background: Lymphangioleiomyomatosis (LAM) is a rare interstitial lung disease characterized by abnormal smooth muscle-like cell (LAM cell) proliferation in the lung stroma. The origin of LAM cells is still unknown. The gold-standard immunohistochemical diagnostic for LAM is an immunopositive reaction to the HMB-45 antibody. Materials and Methods: We aimed to evaluate 15 diagnostic open-lung biopsy specimens of pulmonary LAM. Based on the LAM histologic score (LHS), we distinguished two groups of histological severity: early- and advanced-stage LAM. The expression of HMB-45, estrogen receptor (ER), progesterone receptor (PR), β-catenin, E-cadherin, podoplanin (D2-40), mini-chromosome maintenance protein 3 (MCM3), and epidermal growth factor receptor (EGFR) was evaluated immunohistochemically. Fluorescence in situ hybridization (FISH) was performed in order to investigate amplification of the EGFR gene in LAM cells. Results: The expression of ER and EGFR was significantly higher in advanced than in early-stage LAM. Amplification of the EGFR gene was not detected in any of the 15 studied cases. There was a strong-positive correlation between the expression of PR, ER, β-catenin, E-cadherin, and the standard marker of LAM, HMB45. Conclusion: We conclude that together with LHS, ER may be considered a useful tool for evaluating the progression of LAM. β-Catenin and E-cadherin seem to be new potential specific markers of LAM cells. The increased expression of EGFR in LAM cells is not associated with EGFR gene amplification, although it may be a marker of disease progression; the role of this receptor in LAM pathogenesis should be further investigated. Positive reaction of LAM cells with podoplain demonstrates the existence of an additional lymphatic endothelial lineage in LAM cells.*

Correspondence to: Irmina Grzegorek, M.Sc., Department of Histology and Embryology, Wroclaw Medical University, Chalubińskiego 6a street, 50-368 Wroclaw, Poland. Tel: +48 717841365, Mobile: +48 501647494, Fax: +48 717840082, e-mail: irminagrzegorek@o2.pl

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Pulmonary lymphangioleiomyomatosis (LAM) is a rare interstitial lung disease that predominantly affects women of childbearing age. It is characterized as a low-grade, destructive, metastasizing neoplasm, and manifests as the proliferation of abnormal smooth muscle-like cells in the lung stroma (1). The origin of these cells is still unknown (2). LAM cells are characterized by a mutation in one of two tuberous sclerosis genes: *TSC1* or *TSC2*. LAM may occur as a sporadic form (S-LAM) or may be associated with the tuberous sclerosis complex (TSC-LAM). The gold-standard immunohistochemical diagnostic for LAM is immunopositive reaction with HMB-45 antibody (3). This is a mouse monoclonal antibody that reacts with the GP100 glycoprotein, which is present in small electron-dense granules resembling immature premelanosomes that are characteristic of melanoma cells (4). Besides HMB-45, the most commonly used markers in the immunohistochemical diagnosis of LAM are α-smooth muscle actin (αSMA), estrogen receptor (ER), and progesterone receptor (PR) (5). Individual reports have suggested that β-catenin could be an additional marker of LAM cells and might be involved in LAM pathogenesis (6-8). One protein that is associated with β-catenin is E-cadherin; these act together as an intracellular signal transducer in the WNT signaling pathway (9). E-Cadherin is a type-1 membrane glycoprotein and receptor responsible for cell–cell adhesion. Its dysfunctions are implicated in the promotion of progression of various tumor types (9). E-Cadherin interacts with a range of proteins, and in

this way is involved in intracellular processes, including actin cytoskeleton formation, cell signaling, and trafficking (9). Another protein that may interact with both β-catenin and E-cadherin is epidermal growth factor receptor (EGFR) (9). Additionally, it is very likely that EGFR is significantly involved in LAM. It has been suggested by Lesma *et al.* in their series of studies, that epidermal growth factor (EGF) is a proliferative factor for $TSC2^{-/-}$ smooth muscle cells from human renal angiomyolipoma, and that the blocking of EGFR by monoclonal antibody results in the inhibition of the proliferation of these cells (10, 11). As mentioned, the origin of these cells is still unknown, but it is clear that LAM cells involve both smooth muscle (expression of αSMA) and melanoma (immunoreactivity with antibodies to HMB-45 and CD63, another melanoma marker) lineages (12); the third lineage of lymphatic endothelial cells is still a matter of debate.

In our study, we assessed the histological advancement in 15 diagnostic open-lung biopsy specimens of pulmonary LAM using LAM histological score (LHS). Based on this evaluation, we distinguished a group with early LAM from a group with advanced LAM. We also examined all cases immunohistochemically using standard diagnostic antigens, such as HMB-45, ER, and PR, as well as β-catenin, E-cadherin, podoplanin, EGFR, and minichromosome maintenance complex component 3 (MCM3). The expression and distribution of these antigens in LAM cells were assessed in order to evaluate their usefulness as markers in new diagnostic tools in LAM or as markers of disease progression, or which may shed light on the topic of LAM cell origin.

Materials and Methods

Studied cases. The study was performed on 15 diagnostic open-lung biopsy specimens obtained from individuals in whom the diagnosis of LAM had been confirmed histologically (14 specimens of S-LAM and one specimen of TSC-LAM). The studied group, which is described in Table I, consisted of 15 women with a mean age of 38.9 years (range=24-54 years). Demographic characterization of the group is also presented in Table I.

Tissue samples were collected at the Department of Pathology, National Tuberculosis and Lung Diseases Research Institute, Warsaw and Department of Surgery, Fourth Military Hospital, Wrocław. All specimens were prepared for routine histology using hematoxylin and eosin staining, in line with standard protocols. The histologic severity of LAM was assessed independently by two histopathologists using the LHS (13). The LHS was determined by assessing the total percentage of tissue involvement by cystic lesions and LAM cell infiltration, and was graded as follows: LHS-1, <25%; LHS-2, 25% to 50%; and LHS-3, >50% (13) The study was approved by the Bioethical Committee of the Wrocław Medical University (approval number: KB 594/2012; KB 182/2013).

Immunohistochemistry (IHC). All reagents, antibodies, and apparatus used in the IHC study originate from DakoCytomation, Glostrup, Denmark. For the IHC study, 4-μm-thick paraffin sections were cut. The sections were de-waxed and rehydrated, and the epitopes were exposed using a PT-link instrument, in EnVision™ FLEX Target Retrival Solution, High pH, for 20 min at 97˚C. The IHC reactions were performed in an Autostainer Link 48 using the visualization system of EnVision™ FLEX+, Mouse, high pH. The activity of the endogenous peroxidase was blocked by 5-min exposure to EnVision™ FLEX Peroxidase-Blocking Reagent. Sections were then rinsed with EnVision™ FLEX Wash Buffer and incubated for 20 min at room temperature with the following primary antibodies: MCM-3 (monoclonal, clone 101, M7263, 1:50), HMB-45 [monoclonal, clone HMB-45, M0634; ready to use (RTU)], PR (monoclonal, clone PgR 636; IR068, RTU), ER (monoclonal, clone SP1, IR151; RTU), E-cadherin (monoclonal, clone NCH-38, IR059; RTU), B-catenin (monoclonal, clone β-catenin-1, IR702; RTU), EGFR (monoclonal, clone 2-18C9, K1494; RTU), and podoplanin (monoclonal, clone D2-40, IR072; RTU). The antibodies were diluted in EnVision™ FLEX Antibody Diluent with background reducing component. Secondary goat antirabbit immunoglobulin antibodies were coupled to a dextran core linked to peroxidase. The color reaction was obtained using 3,3’-diaminobenzidine tetrachlorohydrate. All slides were counterstained with EnVision™ FLEX Hematoxylin.

Evaluation of IHC reactions. The IHC sections were evaluated by two independent pathologists using a BX-53 light microscope (Olympus, Tokyo, Japan). The levels of HMB-45, E-cadherin, B-catenin, EGFR, and podoplanin expression were ranked using the modified semi-quantitative Immunoreactive Remmele Score (IRS) of Remmele and Stegner (14). This method takes into account both the proportion of stained cells and the intensity of the reaction, while its final results represent the product of the two parameters, with values ranging from 0 to 12 points (no reaction=0 points; weak reaction=1-2 points; moderate reaction=3-4 points; and intense reaction=6-12 points). Nuclear expression of ER, PR, and MCM3 was scored from 0 to 4 points (no reaction=0 point; 1%-25% of LAM cell nucleus with positive reaction=1 point; 26%-50%=2 points; 51%-75%=3 points; >75%=4 points)

FISH. Interphase FISH was performed on 4-μm-thick formalin-fixed paraffin-embedded tissue sections using the Histology FISH Accessory Kit (Dako, Hamburg, Germany) following the manufacturer’s instructions. To detect gene amplifications or deletions, the *EGFR*/*CEP7* gene/centromere (CEP) probe combinations (Abbott, Wiesbaden, Germany) were used. Hybridization was performed in automated hybridizers (Dako) overnight. For signal detection and enumeration, an Axio Imager Z1 (Zeiss, Oberkochen, Germany) with Isis software (version 5.3.1, MetaSystems, Altlussheim, Germany) was employed. To evaluate the gene copy numbers in each case, the gene- and centromere-specific signals were each counted in 20 non-overlapping tumor cell nuclei. The ratio of the number of gene-specific to centromere-specific signals was calculated. A ratio greater than 2.2 was considered to indicate amplification, and a ratio below 0.8 was considered to show a deletion. Ratios in the range 1.8 to 2.2 and 0.8 to 1.0 were taken as threshold values for amplifications and deletion, respectively. In these cases, 20 more nuclei were enumerated in order to obtain an unequivocal value.

Statistical analysis. Statistical analysis was performed using Prism 5.0 (GraphPad, La Jolla, CA, USA). The Mann–Whitney *U*-test and

the Kruskall–Wallis test with post hoc Dunn's multiple comparison test were used to compare the groups of data that failed to satisfy the assumptions of the parametric test. Correlations between the scores of the examined IHC markers were tested using Spearman's correlation test. In all these analyses, results were considered statistically significant for $p<0.05$.

Results

H&E staining of 15 cases of pulmonary LAM showed lung tissue with thin-walled cysts filled with LAM cells and LAM nodules with the typical arrangement of cells. Smaller spindle-shaped smooth-muscle-like cells were located centrally and surrounded by larger epithelioid-like cells. The histological severity of LAM was assessed using the LHS. Of the 15 studied cases, eight (53.3%) were evaluated as LHS-1. A total of five out of the 15 cases (33.3%) had LHS-2, and two out of the 15 (13.3%) had LHS-3 scores. Based on this evaluation, the study population was divided into two groups: early-stage LAM (LHS-1; eight cases) (Figure 1A) and advanced-stage LAM (LHS-2 and -3; seven cases) (Figure 1B). Positive immunohistochemical reaction with the HMB-45 antibody (Figure 2A) was observed in all 15 (100%) studied cases, for both types of LAM cells. From the 15 cases, three (20%) showed weak, six (40%) moderate, and six (40%) intense reaction with this antibody. However, there were no statistically significant differences observed in expression between the early-stage and advanced-stage LAM groups. Expression of ER (Figure 2C) and PR (Figure 2B) was observed in all 15 (100%) cases, mostly in spindle-shaped smooth muscle-like cells. However, stronger immunoreactivity was generally noted for PR. The expression of ER was weak in 8 out of 15 cases (53.3%), moderate in 4 out of 15 (26.6%), and strong in 3 out of 15 (20%) cases, and was significantly higher in the group with advanced-stage LAM (mean IRS=2.14±0.69) than in the group with early-stage LAM (mean IRS=1.25±0.71) ($p<0.05$). The expression of PR was weak in 5 out of 15 cases (33.3%), moderate in 3 out of 15 (20%), strong in 5 out of 15 (33.3%), and very strong in 2 out of 15 cases (13.3%). We did not observe any significant differences between the studied groups.

In all 15 cases, we observed cytoplasmic and membranous expression of β-catenin (Figure 2E). The IHC staining was weak in six out of 15 cases (40%), moderate in two out of 15 (13.3%), and intense in seven out of 15 (46.6%), but there were no significant differences in the expression of β-catenin between early-stage and advanced-stage LAM. Expression of E-cadherin (Figure 2F) was shown in all 15 cases (100%) in the cytoplasm and cell membrane of both spindle-shaped smooth muscle-like and epithelioid-like cells. The intensity of E-cadherin staining was moderate in one out of 15 cases (6.6%) and strong in 14 out of 15 cases (93.3%). No differences in expression between the two groups were observed.

Table I. *Demographic characterization of the study group.*

Case	TSC	Age, years	Additional disorders
1	–	43	Uterine myoma
2	–	40	Epilepsy
3	–	40	Liver cysts
4	–	28	Retroperitoneal lymphangioma
5	–	43	–
6	–	46	Irritable bowel syndrome, stomach polyps
7	–	48	Hyperthyreosis
8	+	24	Brain tumor, kidney tumor, epilepsy, blindness
9	–	47	–
10	–	43	Hypothyreosis
11	–	44	–
12	–	40	–
13	–	43	–
14	–	54	Limited scleroderma, multinodular goiter
15	–	42	–

LAM, Lymphangioleiomyomatosis; TSC, Tuberous Sclerosis Complex.

Diffuse, cytoplasmic expression of podoplanin (D2-40), a marker of lymphatic endothelium, was shown in all 15 cases (100%) in the LAM cells and in small lymphatic vessels present in the LAM nodules (Figure 2H). Immunohistochemical staining for D2-40 was present in most of the LAM cells in LAM nodules, but was weaker than in the lymphatic endothelial cells. Intensity of D2-40 staining ranged from weak in 9 out of 15 cases (60%), moderate in 3 out of 15 (20%), and strong in 3 out of the 15 LAM cases (20%), but did not differ between the groups. Expression of MCM3 was observed in individual LAM cells (Figure 2D), being classified as a weak reaction in all 15 cases (100%) at the same level.

Expression of EGFR was observed in 8 of 15 cases (53.3%) and was shown to be weak in 7 out of 15 (46.6%) and moderate in 1 of 15 (6.6%) cases. Cytoplasmic and membranous staining of EGFR was present only in some of the LAM cells in LAM nodules. However, expression of EGFR (Figure 2G) was significantly higher in advanced-stage LAM (mean IRS=1.86±1.2) than in early-stage LAM (mean IRS=0.37±0.74) ($p<0.05$).

The semi-quantitative scoring of all the immunohistochemical reactions is shown in Table II and Spearman correlations between the expression of the markers are presented in Table III.

In order to investigate whether elevated levels of EGFR expression in advanced-stage LAM are associated with amplification of *EGFR* gene, FISH was performed on all 15 LAM cases. Amplification of *EGFR* gene was not detected in any of the 15 studied cases. Polysomy of chromosome 7 and *EGFR* (mostly trisomy or tetrasomy) was seen in some LAM cells in all studied cases, but did not constitute the major population of LAM cells in LAM nodules.

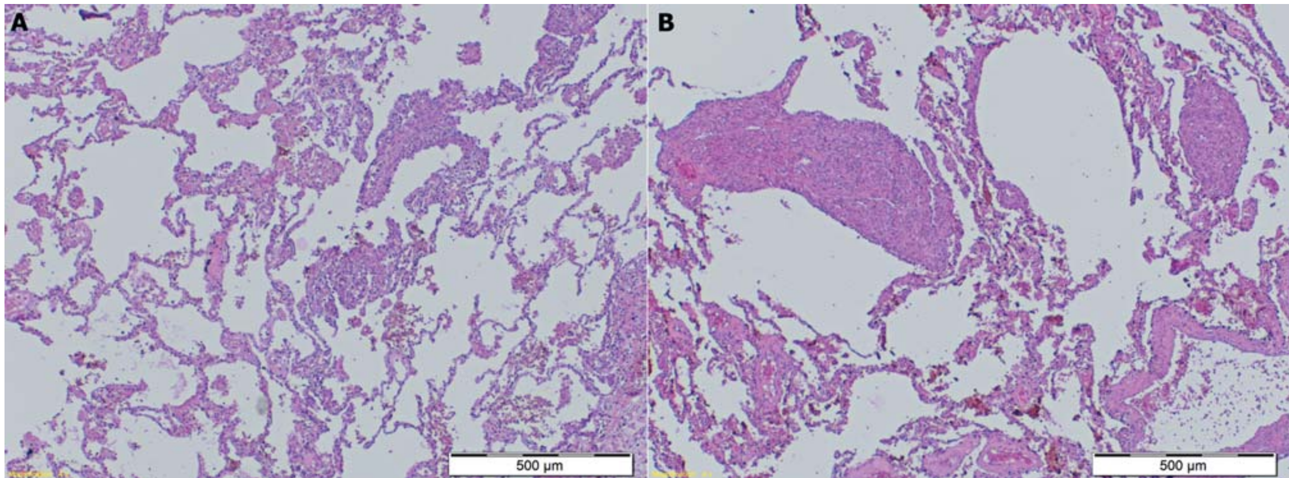

Figure 1. *Hematoxylin-eosin staining of open lung biopsy specimens representing early-stage lymphangioleiomyomatosis (LAM) (LHS-1) (A) and advanced-stage LAM (LHS-3) (B).*

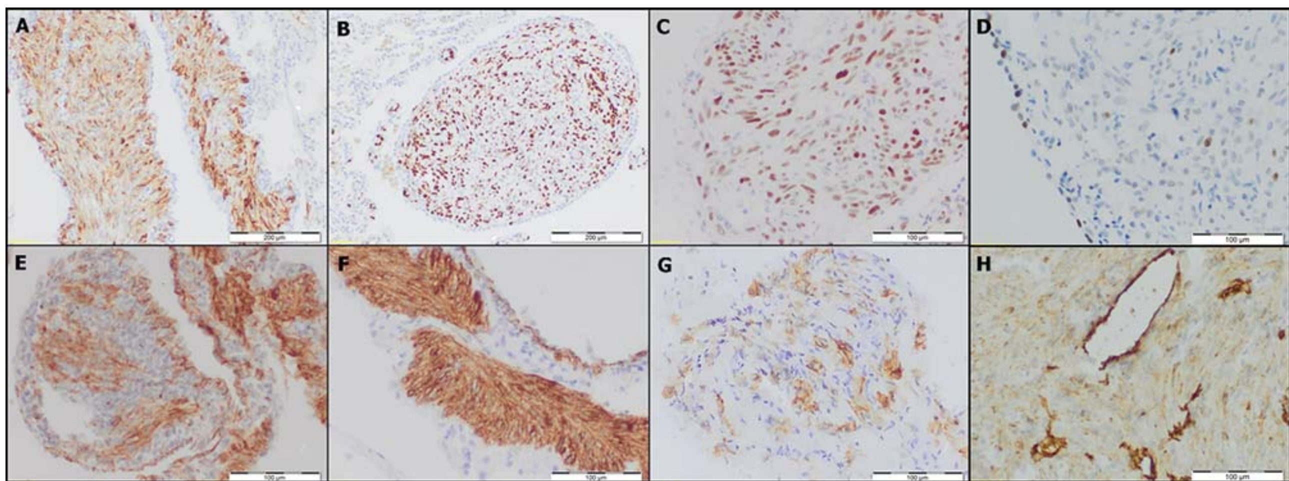

Figure 2. *Immunohistochemical staining of lymphangioleiomyomatosis (LAM) with HMB-45 (A), progesterone receptor (PR) (B), estrogen receptor (ER) (C), mini-chromosome maintenance protein 3 (MCM3) (D), β-catenin (E), E-cadherin (F), epidermal growth factor receptor (EGFR) (G), and podoplanin (D2-40) (H).*

Discussion

The histological and immunohistochemical evaluation of lung biopsy specimens is the basis of diagnosis of pulmonary LAM. The most common markers used in LAM IHC diagnostics are HMB-45, αSMA, PR and ER. In our study, we decided to evaluate 15 open-lung biopsy specimens by IHC with both commonly used diagnostic markers, such as HMB-45, PgR and ER, and other antigens (β-catenin, E-cadherin, podoplanin, MCM3, EGFR) that could be considered as new diagnostic markers in LAM. The expression of these markers was in all cases estimated according to their histological advancement, as evaluated using the LHS. All the results were tested for correlation with each other in order to estimate the usefulness of particular antigens or their combination in LAM diagnostics.

HMB-45 has high specificity in the diagnosis of LAM in contrast to other cystic pulmonary diseases (15). However, not all LAM cells exhibit reactivity for HMB-45 and rare LAM cases have been shown to be HMB-45-negative (16). It has been reported that immunoreactivity for HMB-45 is more frequent for the epithelioid type of LAM cell (2). In

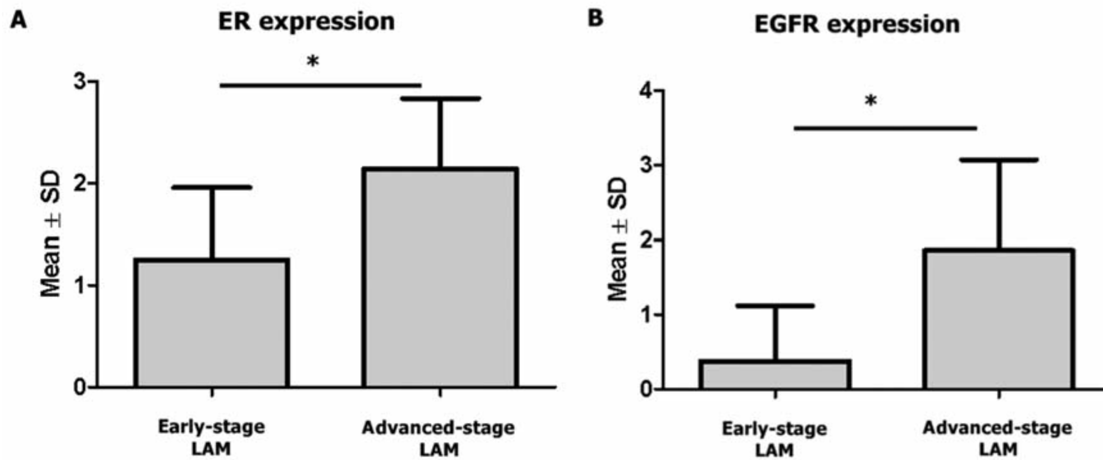

Figure 3. *Expression of estrogen receptor (ER) and epidermal growth factor receptor (EGFR) in early and advanced stages of LAM. *p<0.05.*

Table II. *Semi-quantitative scoring of immunohistochemical reactions. LHS, LAM histological score; HMB-45, human melanoma black 45; PR, progesterone receptor; ER, estrogen receptor; D2-40, podoplanin; MCM3, mini-chromosome maintenance protein 3; EGFR, epidermal growth factor receptor.*

	Score								
Case	LHS	HMB-45	PR	ER	β-Catenin	E-Cadherin	D2-40	MCM3	EGFR
1	2	6	2	2	4	6	6	1	4
2	1	6	3	3	6	9	4	1	0
3	1	2	1	1	1	6	2	1	0
4	1	3	1	1	1	6	4	1	0
5	1	9	3	1	9	9	3	1	1
6	1	3	1	1	4	4	1	1	0
7	3	3	3	2	6	12	2	1	0
8	1	3	2	1	2	6	6	1	0
9	2	4	2	2	6	12	2	1	2
10	2	6	4	3	8	9	8	1	2
11	2	6	3	3	9	9	2	1	2
12	1	3	3	1	2	9	2	1	2
13	3	12	4	2	6	9	2	1	1
14	1	1	1	1	2	6	2	1	0
15	2	1	1	1	1	6	2	1	2

Table III. *Spearman correlation between the expression of the investigated antigens. LHS, LAM histological score; HMB-45, human melanoma black 45; PR, progesterone receptor; ER, estrogen receptor; D2-40, podoplanin; MCM3, mini-chromosome maintenance protein 3; EGFR, epidermal growth factor receptor.*

	LHS	HMB-45	PR	ER	B-Catenin	E-Cadherin	D2-40	EGFR
LHS	x	r=0.34, p=0.22	r=0.46, p=0.08	r=0.6, p=0.02	r=0.4, p=0.14	r=0.5, p=0.05	r=–0.07, p=0.8	r=0.49, p=0.06
HMB-45		x	r=0.77, p<0.001	r=0.63, p=0.01	r=0.8, p=0.0003	r=0.5, p=0.06	r=0.36, p=0.18	r=0.36, p=0.17
PR			x	r=0.67, p=0.006	r=0.76, p=0.0008	r=0.75, p=0.001	r=0.28, p=0.3	r=0.33, p=0.2
ER				x	r=0.7, p=0.004	r=0.6, p=0.02	r=0.27, p=0.3	r=0.35, p=0.2
B-Catenin					x	r=0.67, p=0.006	r=0.12, p=0.7	r=0.29, p=0.3
E-Cadherin						x	r=0.01, p=0.9	r=0.28, p=0.3
D2-40							x	r=0.14, p=0.6
EGFR								x

our study, all LAM cases were HMB-45-positive. Immunoreactivity was shown in both types of LAM cells, but not all LAM cells were stained by this antibody.

The expression of ER and PR receptors was observed in nuclei of both types of LAM cells in all studied cases, with higher frequency for PR than for ER, which is consistent with the observations of other groups (17-19). The expression of both of these receptors is strongly correlated with the expression of HMB-45. Interestingly, we observed significantly higher ER receptor expression in the group with advanced-stage LAM than in the group with early-stage LAM. Additionally, the strong positive correlation between LHS and the expression of ER suggests that ER expression in LAM cells increases together with the severity of the disease. ER receptors could be considered not only as diagnostic markers of LAM, but, together with LHS, also as a useful tool for evaluating the progression of the disease.

In all investigated cases, we observed a high expression of β-catenin; however, we did not note any significant differences in the expression levels between early and advanced LAM. The high specificity of β-catenin for LAM cells and the strong positive correlations between the expression of β-catenin and HMB-45, PR, and ER suggest that β-catenin could be an additional useful diagnostic marker of LAM cells. Our results are consistent with the study of Flavin *et al.*, where the expression of β-catenin in LAM cells of 28 pulmonary LAM and 10 renal angiomyolipoma cases was presented (6). Other studies suggest that β-catenin plays a role in LAM pathogenesis (20, 7, 8). Barnes *et al.* showed evidence that the loss of tuberin promotes LAM cell invasion through the β-catenin pathway (7, 8). They also showed that tuberin-null invasive cells express cleaved forms of β-catenin, which are in turn transcriptionally active and promote expression of matrix metalloproteinase-7 (MMP7) (7, 8).

The expression of E-cadherin was present in all the cases studied here, without significant differences in the different stages of advancement of LAM; however, there was a positive correlation between its expression and the LHS. Additionally, the expression of E-cadherin correlated strongly with HMB-45, PR, ER and β-catenin, which allows E-cadherin to be considered an additional diagnostic marker of LAM. It has been shown in *in vitro* studies that *TSC2*-null cells exhibit loss of membrane E-cadherin and undergo the epithelial–mesenchymal transition, which is characterized by decreased levels of E-cadherin expression and its re-localization to cytosolic structures, as well as the increased expression of the markers of the epithelial–mesenchymal transition, namely, zinc finger protein SNAI1 (SNAIL) and αSMA (7, 8, 21).

The lymphatic endothelial differentiation of LAM cells is still controversial. Hansen *et al.*, in their IHC study, reported no expression of podoplanin in LAM cells. Immunoreactivity with D2-40 was present only in thin-walled lymphatic vessels accompanying LAM lesions (22). Contrary to these results, a recent study showed D2-40-positive IHC reaction in the majority of LAM cells in all studied cases of sporadic LAM, with no differences between early-stage and late-stage cases (23). The expression of other lymphatic-specific markers, such as prospero homeobox protein 1 (PROX1), vascular endothelial growth factor receptor 3 (VEGFR3) and lymphatic vessel endothelial receptor 1 (LYVE1), has been shown in LAM cells, which provides evidence that in sporadic LAM, LAM cells may have an additional lineage of lymphatic endothelial differentiation (23). In our study, we obtained results that support these more recent conclusions (23). We observed diffuse, cytoplasmic expression of podoplanin in all our cases, without any significant differences between early and advanced LAM. However, the intensity of IHC staining in the LAM cells was weaker than in the lymphatic endothelial cells. In our group's previous study, we showed positive immunofluorescence staining for D2-40 in a cell line cultured from the chylous effusion of a 42-year-old female patient with sporadic pulmonary LAM (case no. 15) (24). There were no correlations between the expression of podoplanin and the other studied markers. The role of lymphatic differentiation in LAM cells should be further examined.

The expression of markers of proliferation, such as proliferating cell nuclear antigen (PCNA) and Ki-67, in LAM cells has been described mostly in smooth muscle-like LAM cells and inversely correlated with the expression of HMB-45 (25, 26). However, fewer than 20% of the total population of LAM cells are immunoreactive with Ki-67 antigen (12). MCM proteins have been shown to be better, more specific markers of proliferation than Ki-67 or PCNA in various types of carcinomas (27). In our study, we examined the antigen MCM3 as a proliferative marker for LAM cells. However, the expression of MCM3 was observed only in individual LAM cells (fewer than 10% of all the LAM cells), and was classified as a weak reaction in all cases on the same level.

The role of EGFR in pulmonary lymphangioleio-myomatosis is still not clear. Lesma *et al.*, in their series of studies, showed that EGF is a proliferative factor for $TSC2^{-/-}$ smooth muscle cells from human renal angiomyolipomas (10, 11). The blocking of EGFR by monoclonal antibodies resulted in inhibition of their proliferation, the reversion of their immunoreactivity to HMB-45, and the inhibition of the constitutive phosphorylation of S6 and extracellular-signal-regulated kinase (ERK) in an *in vitro* model (11). Additionally, the administration of monoclonal antibody to EGFR in the mouse model of LAM resulted in a reduction in the number of $TSC2^{-/-}$ smooth muscle cells and blood vessel proliferation (28). In our study, we examined the expression

of EGFR in 15 cases and found that the expression of this receptor in LAM cells is very heterogeneous. The expression of EGFR was observed only in 8 out of 15 cases and increased together with disease advancement. The expression of EGFR was significantly higher in advanced-stage than in early stage LAM. Additionally there was a positive Spearman's correlation between EGFR expression and LHS. We conclude that EGFR may have an important role in LAM pathogenesis, especially in the more advanced stages. However, this phenomenon is not associated with gene amplification because amplification of EGFR was not detected in any of the 15 studied cases.

In summary, our immunohistochemical evaluation of pulmonary LAM has shown that ER, already used as a standard diagnostic marker of LAM, together with LHS could also be a useful tool for evaluating the progression of LAM. β-Catenin and E-cadherin, which strongly correlated with HMB-45, ER and PR, seem to be new specific markers of LAM cells that could be used as additional diagnostic tools for lung biopsies. The expression of podoplanin in LAM cells demonstrates the existence of an additional lymphatic endothelial lineage of LAM cells, in addition to their smooth muscle and melanoma lineage. The increasing expression of EGFR in LAM cells is not associated with amplification of *EGFR* gene, but may be a marker of disease progression; the role of this receptor in LAM pathogenesis should therefore be further investigated.

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