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ORIGINAL ARTICLE

In pulmonary lymphangioleiomyomatosis expression of progesterone receptor is frequently higher than that of estrogen receptor

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Abstract Lymphangioleiomyomatosis (LAM) of the lung is a rare low-grade malignancy affecting primarily women of childbearing age. LAM is characterized by the proliferation of SMA and HMB-45 positive spindle-shaped and epithelioid cells throughout the lung in the form of discrete lesions causing cystic destruction and ultimately respiratory insufficiency. LAM occurs sporadically or in patients with tuberous sclerosis complex (TSC) and is etiologically linked to mutations in the *TSC1* and *TSC2* genes. Although LAM cells are known to express estrogen and progesterone receptors (ER and PR, respectively), their respective expression level was never determined. Therefore, here we measured the immunohistochemical expression of ERs and PRs in a large series of pulmonary LAM cases using the Aperio Spectrum Analysis Platform. Our case series comprised open lung biopsy specimens from 20 LAM patients and lungs explanted during the course of lung transplant from 24 patients. All cases were positive for ER and PR. PR expression was statistically significantly higher than ER in 80 % of the biopsies while ER predominated only in one case. Specimens from explanted cases of LAM had relatively fewer PR-positive nuclei. As a result, PR expression was significantly higher than ER in 38 % of the cases, whereas ER predominated in 33 %. Overall, PR expression predominated in 57 % of cases and ER in 21 %. These data indicate that PR frequently prevails over ER in pulmonary LAM. LAM is unusual in its high PR/ER ratio; other female neoplasms show a definite prevalence of ER. Our findings therefore warrant further study of PR function in LAM.

Keywords Lymphangioleiomyomatosis · Estrogen receptor · Progesterone receptor · Biopsy · Explanted lung

Introduction

Lymphangioleiomyomatosis (LAM) of the lung is a rare disease affecting mainly women of reproductive age [1]. LAM is characterized by the proliferation of abnormal cells (LAM cells) throughout the lung, forming discrete lesions which lead to cystic destruction, pneumothorax, chylous pleural effusion, and ultimately respiratory insufficiency [2–4]. Studies have provided strong evidence that LAM cells are neoplastic and have metastatic potential [5–7]. LAM cells are largely spindle-shaped, with a small proportion of epithelioid cells [8], and are characterized immunohistochemically by expressing smooth muscle actin (SMA) and human melanoma black-45 (HMB-45, gp100), a melanocytic marker [9–12]. LAM occurs sporadically (S-LAM) or in patients with tuberous sclerosis complex (TSC-LAM), an autosomal dominant genetic disorder characterized by tumors in the brain, heart, skin, kidneys, and other organs and often marked by mental retardation or seizures [13–15]. Since the first S-LAM case was reported in 1937 [4], it has become evident that the majority of LAM patients who seek medical attention suffer from S-LAM. Currently, lung transplantation is the only effective treatment for end-stage LAM [16, 17], but recurrence after transplant can occur [6, 18].

It has been shown that mutations in *TSC1* (*tuberous sclerosis complex 1*) or *TSC2* (*tuberous sclerosis complex 2*) are a critical factor in the etiology of LAM [19, 20]. Their protein products, referred to as hamartin, or TSC1, and tuberin, or

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TSC2, form a tumor suppressor dimer [21, 22] that inhibits mTORC1 (mammalian target of rapamycin complex 1) by directly inhibiting the activity of the small GTPase Rheb (rho enriched in brain) via the GAP (GTPase-activating protein) domain of TSC2 [23–26]. The mTOR pathway is critical in the control of cell growth, proliferation, and metabolism [27]. Rapamycin, an mTOR inhibitor, is currently used to treat LAM patients. The drug has been shown to induce marked improvement of pulmonary functions in approximately 50 % of LAM patients [28–30]. However, cessation of rapamycin therapy resulted in return to the diminished pulmonary function observed prior to the treatment [28, 29].

The fact that LAM affects women of reproductive age and exacerbates during pregnancy [31, 32] and contraceptives intake [33–35], coupled with the observation that LAM lesions generally express estrogen receptor (ER) and progesterone receptor (PR) [36–39], has long suggested a role for female reproductive hormones in the development of the disease. Such notion is supported by animal experimental evidence showing that estrogens promote the survival and growth of tuberin-null cells implanted in nude mice [40]. On the assumption that estrogen plays a role in the progression of LAM, treatments had been aimed at reducing its effects, either directly with anti-estrogen drugs, such as tamoxifen, or indirectly, by counterbalancing the effects of estrogen with the administration of progestins [41–46]. However, the results of such therapeutic approaches were generally discouraging. Interestingly, the potential role of PRs in LAM has not been studied, neither have treatments aimed at reducing their effect been undertaken.

Published studies of ER and PR expression in LAM have been restricted to few cases or confined to extrapulmonary LAM [34, 36–39, 47, 48], and none of them included receptor quantification. Since the quantification of ER and PR expression represents a basic step towards gaining a better understanding of their role in LAM, here we applied quantitative image analysis (QIA) to a large series of LAM cases to obtain such information.

Materials and methods

Case selection

After obtaining approval from the Institutional Review Board (IRB) of the University of Chicago Biological Sciences Division, consent forms along with a query about hormonal medications and pulmonary test function at the time of biopsy were sent to individual patients who had a diagnosis of LAM made by open lung biopsy and agreed to participate in the study. Unstained formalin-fixed and paraffin-embedded lung sections were received from 20 of these patients, 19 of them with S-LAM, and 1 with genetically confirmed TSC-LAM. All biopsies were fixed immediately and processed overnight. Formalin-fixed and paraffin-embedded lung tissue samples from 23 S-LAM patients and one TSC-LAM patient were obtained from the National Disease Research Interchange (NDRI). All samples were procured from lungs removed at the time of lung transplant, fixed immediately, and embedded the next day.

Immunohistochemistry

Mouse monoclonal antibodies (Abs) against PR (clone no. 312) and ER (clone no. 6F11) were obtained from Leica Microsystems (Buffalo Grove, IL). An additional set of mouse monoclonal Abs against PR and ER were obtained from Dako (Carpinteria, CA) and used in selected cases to compare ER and PR quantification obtained using different brands of Abs. Mouse monoclonal Abs against smooth muscle actin (SMA, M0851) and human melanosome (HMB-45, M0634) were purchased from Dako. Five 4–5 μm consecutive formalin-fixed, paraffin-embedded sections from each case (one to three slides per case) were respectively immunostained for SMA, HMB-45, PR, and ER, and the last section was stained with hematoxylin-eosin (H&E). The Abs were used at the following dilutions: anti-both anti-ER Abs, 1:100; both anti-PR Abs, 1:200; anti-SMA Ab, 1:100; and HMB-45 Ab, 1:50. Immunohistochemistry (IHC) for ER, PR, and HMB-45 was performed on a Leica Bond-Max automated IHC/ISH system (Leica Microsystems), according to a modified manufacturer's protocol, using Bond™ Polymer Refine detection (DAB) system (Leica Microsystems). Briefly, following heat antigen retrieval (no pretreatment for HMB-45), sections were incubated with each antibody for 25 min, followed by post-primary step for 15 min and Bond polymer HRP for 25 min, and then by peroxidase block for 5 min. The peroxidase reaction was developed using 3,3 diaminobenzidine (DAB) provided in the kit, followed by counterstaining with hematoxylin for 5 min. Slides were dehydrated in alcohols and mounted in mounting medium (Sakura Finetek, Torrance, CA). IHC for SMA was performed on a Benchmark XT instrument using the UltraView HRP Universal Detection kit (Ventana Medical Systems, Tucson, AZ) according to the manufacturer's protocol.

Double IHC was performed on a Leica Bond-Max automated IHC/ISH system (Leica Microsystems Inc). The sections first underwent immunostaining with anti-ER or anti-PR Ab, as described above, followed by a second round of antigen retrieval and incubation with anti-SMA or HMB-45 Ab, and then processed with the Bond Polymer Red detection system (alkaline phosphatase) (Leica Microsystems) according to the manufacturer's protocol.

All appropriate controls were performed. Human breast cancer sections served as positive control for anti-PR and anti-ER Abs; melanoma sections served as positive control

for HMB-45 Ab; lung blood vessel, and bronchial smooth muscle served as a positive control for SMA Ab; and non-LAM lung sections served as negative controls. All slides were examined by three board-certified pathologists (LG, EH, and LS).

Digital imaging and quantitative image analysis

The slides, single immunostained for PR and ER and double immunostained for PR, ER, and SMA, were scanned using the Aperio ScanScope (Aperio technologies, Vista, CA, USA). A minimum of 10 random areas of LAM lesions per case, equivalent to over 40 high-power fields/case, were selected for quantification of ER and PR expression. Aperio ImageScope software (Aperio Technologies) with nuclear quantification algorithms that were specifically designed for single or double immunostaining were used in this study (supplementary Table S1); the algorithms were based on the spectral differentiation between brown (positive) and blue (negative) staining. Quantitative scores of immunostaining intensities of +1, +2, +3, and total percentage of positivity (sum of 1+, 2+, and 3+) were recorded for each case. Statistical analysis was performed using the student *t* test and a *p* value of 0.05 or less was considered significant. The percentage of total stained PR-positive and ER-positive nuclei absorption was also calculated and shown by the optical density (OD) [OD=log10 (240/average positive intensity)] according to the instructions of Aperio program.

Confocal immunofluorescence microscopy

Two explanted cases of LAM were double immunostained with HMB-45 and anti-ER or anti-PR Abs and studied by confocal microscopy. Double confocal immunofluorescence microscopy (IFC) was performed on 5 μm frozen tissue sections. All appropriate controls were performed. Sections were fixed in 2 % paraformaldehyde for 15 min. After permeabilizing in 0.5 % IGEPAL CA-630 (Sigma-Aldrich, St. Louis, MO) for 5 min, the sections were incubated at room temperature for 3 h with anti-PR Ab (1:200) or anti-ER Ab (1:100). The sections were then treated for 1 h with the secondary Ab at a 1:1,000 dilution (Alexa Fluor 594 goat anti-mouse IgG H+L, catalog no. A-11005, Molecular Probes, Life Technologies, Grand Island, NY). Afterwards, the sections were exposed to HMB-45 Ab for 2 h at room temperature, followed by incubation for 1 h with the secondary Ab at a dilution of 1:1,000. Each step was followed by three washes in PBS for 5 min each. Micrographs were taken under a Zeiss spinning disk inverted confocal fluorescence microscope using SlideBook software (Intelligent Imaging Innovations, Denver, CO).

Results

The diagnosis of LAM was confirmed by microscopic examination of serial sections stained with H&E, anti-SMA Ab, and HMB-45 Ab. LAM lesions were mostly composed of spindle cells with scattered epithelioid cells. The large majority of the LAM cells were positive for SMA. Cells positive for HMB-45 were less numerous and varied from case to case. Only in few lung biopsies the LAM lesions were significantly smaller than the rest; otherwise, the lesion size appeared similar in diagnostic biopsies compared with the specimens from explanted cases of LAM. The major difference rested in the size and number of the cysts, which was far more prominent in the specimens from explanted cases of LAM.

All 44 cases studied were positive for ER and PR, and no significant differences in number of positive nuclei or intensity of immunoreactivity were detected between the Leica and the Dako Abs in the selected cases immunostained with both Abs (not shown). ER and PR expression was confined to LAM cells, as identified by their morphology, as well as their positivity for SMA and/or HMB-45. Although the number of ER- and PR-positive nuclei varied greatly from case to case (Fig. 1), it was highly consistent among lesions within each case (not shown).

Nineteen out of the 20 lung biopsies studied (90 % of the cases) showed a higher percentage of PR-positive nuclei compared to that of ER-positive nuclei when quantifying total positivity (Fig. 1a), or when quantifying +1, +2, and +3 intensities individually (supplementary Figure 1). In 16 out of these 19 cases, the difference was statistically significant, but in the other three (cases no. 6, 11, and 13) the difference was small (Fig. 1a), consistent with the data shown by absorption (Fig. 1b). The findings were essentially the same when LAM cells were identified solely by their morphology (Fig. 2a, b) or when identified by their positivity for SMA in double immunostained sections (Fig. 2c, d). Double immunofluorescence coupled with confocal microscopy confirmed the presence of ER and PR in HMB-45-positive cells (Fig. 3).

Twelve out of 24 explanted cases of LAM showed a higher percentage of PR-positive nuclei compared to that of ER, and this difference was statistically significant in nine cases (Fig. 4a). In 11 out of the other 12 cases, ER expression predominated over PR, and the difference was statistically significant in eight cases.

The overall average of PR expression in LAM biopsies was higher than that of ER ($p=0.00027$); however, there was no statistically significant difference between the two receptors in explanted cases of LAM ($p=0.25$), or between diagnostic biopsies and explanted cases of LAM ($p=0.17$) (Fig. 5).

In summary, from the 44 cases studied, 25 showed a statistically significant predominance of PR, 9 showed a statistically significant predominance of ER, and 10 showed no

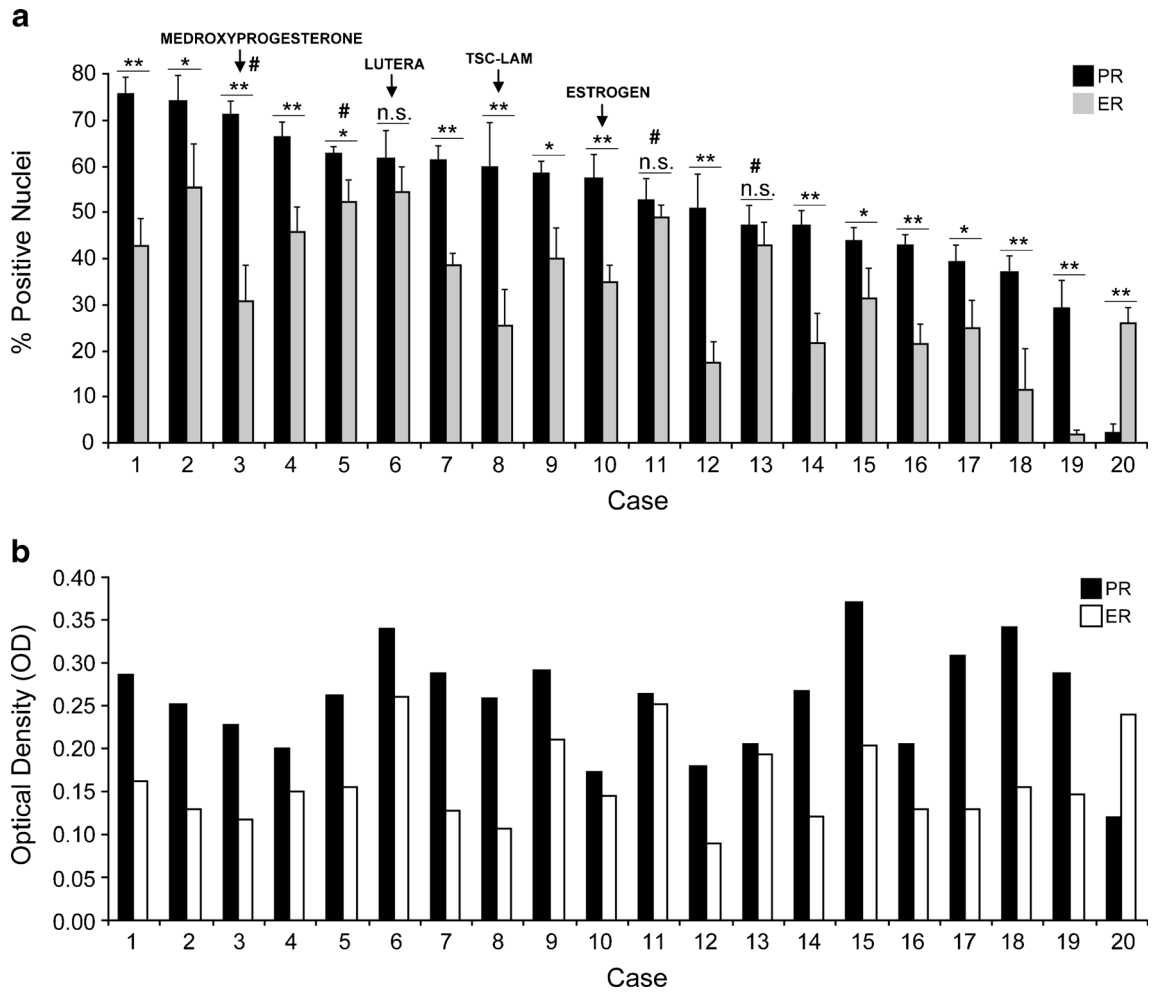

Fig. 1 Quantitative image analysis of PR and ER expression in biopsy cases of LAM (n=20). **a** Percentage of total PR-positive and ER-positive nuclei per case. Data represent mean±SD. **p<0.01, *p<0.05 and *n.s.*, non-significant by one-way ANOVA. # represents cases with abnormal lung function tests. **b** Percentage of total stained PR-positive and ER-positive nuclei absorption is shown in log scale by *optical density* (*OD*) [OD=log10 (240/average positive intensity)]

statistical significance differences between the two receptors (Fig. 6).

Discussion

Lymphangioleiomyomatosis (LAM) of the lung is a rare low-grade neoplasia characterized by the development of LAM lesions and cysts [1] with underlying *TSC2* or *TSC1* mutations which lead to activation of the mTOR pathway [19, 20]. Although *TSC* mutations play an essential role in the pathogenesis of LAM, studies have shown that not all LAM cases have *TSC* mutations [49], and not all TSC patients develop LAM [7], indicating that additional factors may contribute to the development of the disease.

Several studies reported ER and PR expression in LAM cells [34, 36–39, 47, 48, 50], suggesting that their growth is, at least in part, hormone-related. However, hormonal therapies targeting the ER, including progesterone and anti-estrogen drugs, used in the past to treat the disease [41–46], met with disappointing results.

Interestingly, it has been demonstrated that estrogens promote the survival and growth of rat-derived TSC2-null cells implanted in nude mice [40], a finding at odds with the outcome of hormonal therapies aimed at neutralizing the effects of estrogens. In trying to understand why such therapies were mostly unsuccessful, we reviewed the studies published on PR and ER in LAM [34–39, 47] and found that none of them provided a quantitative assessment for the number of LAM cells positive for each receptor to determine their expression relative to each other. To fill this basic gap in information, we used QIA to analyze a total of 44 LAM lung specimens comprising 20 diagnostic open lung biopsies and 24 explanted LAM specimens obtained during lung transplant for explanted cases of LAM.

We found that all cases in our series were positive for PR and ER with a wide range of variability between cases, but with little or no variability between lesions within the same

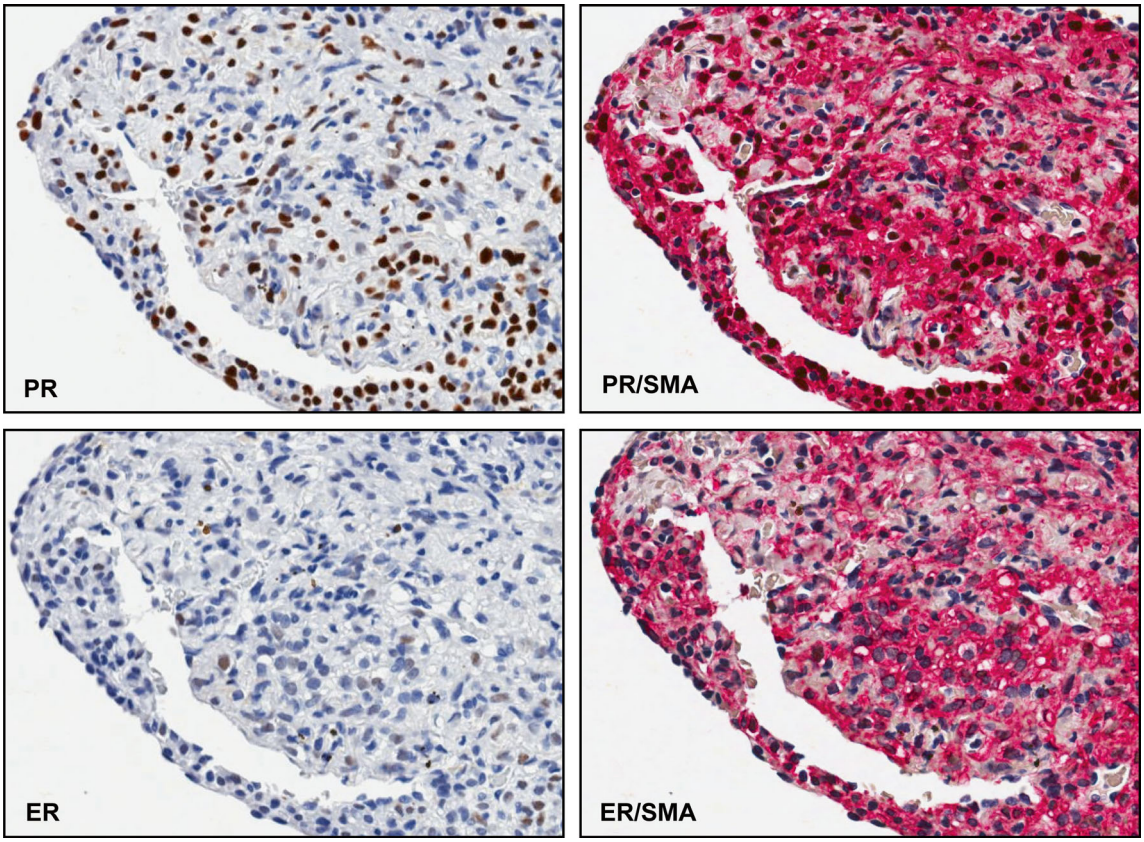

Fig. 2 Immunohistochemical study of PR and ER expression in representative biopsy cases of LAM. Single and double IHC stains with accompanying H&E stain (×10)

case. In two explanted cases of LAM, the expression of both receptors was very low (less than 5 % of the LAM cells), which is consistent with literature documenting negative cases, especially for ER [34, 36, 47]. Receptor expression was confined to LAM cells, as identified by their morphology, as well as their expression of SMA and/or HMB-45. Several reports indicated that PR and ER expression is restricted to LAM cells displaying epithelioid morphology [5, 35, 41]. Our results, however, do not support such a notion, as PR and ER expression was abundantly detected in spindle-shaped LAM cells as well as in some, but not all epithelioid, HMB-45 positive cells.

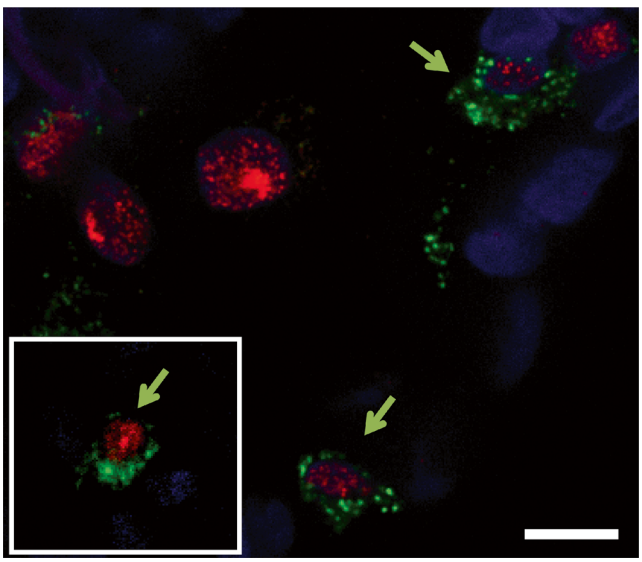

Fig. 3 Confocal double IFC analysis on a representative explanted case of LAM using anti-PR (*red*) and HMB45 (*green*) Abs. Nuclei were stained with DAPI (*blue*). The *arrow points* to LAM cells with colocalization of PR and HMB45 (×100; *lower left insert*, ×40). *Scale bar*, 20 μm

QIA analysis showed that in 25 cases (57 % of total cases) comprising 16 diagnostic biopsies (80 % of all biopsies) and in 9 explanted cases of LAM samples (38 % of all explanted cases of LAM samples), PR expression predominated over ER in a statistically significant manner regardless of the Ab brand used to detect them. This difference in receptor expression was unrelated to the age of the patient and occurred regardless of whether or not the patients received hormonal therapy (progesterone, estrogen, or both), which has been previously reported to eliminate ER and PR receptor expression in patients from whom biopsy material was obtained before and after receiving hormonal therapy [48].

Ten cases (23 % of total cases) comprising three diagnostic biopsies (15 % of all biopsies) and seven explanted cases of LAM specimens (29 % of all explanted cases of LAM specimens) showed no statistically significant difference between

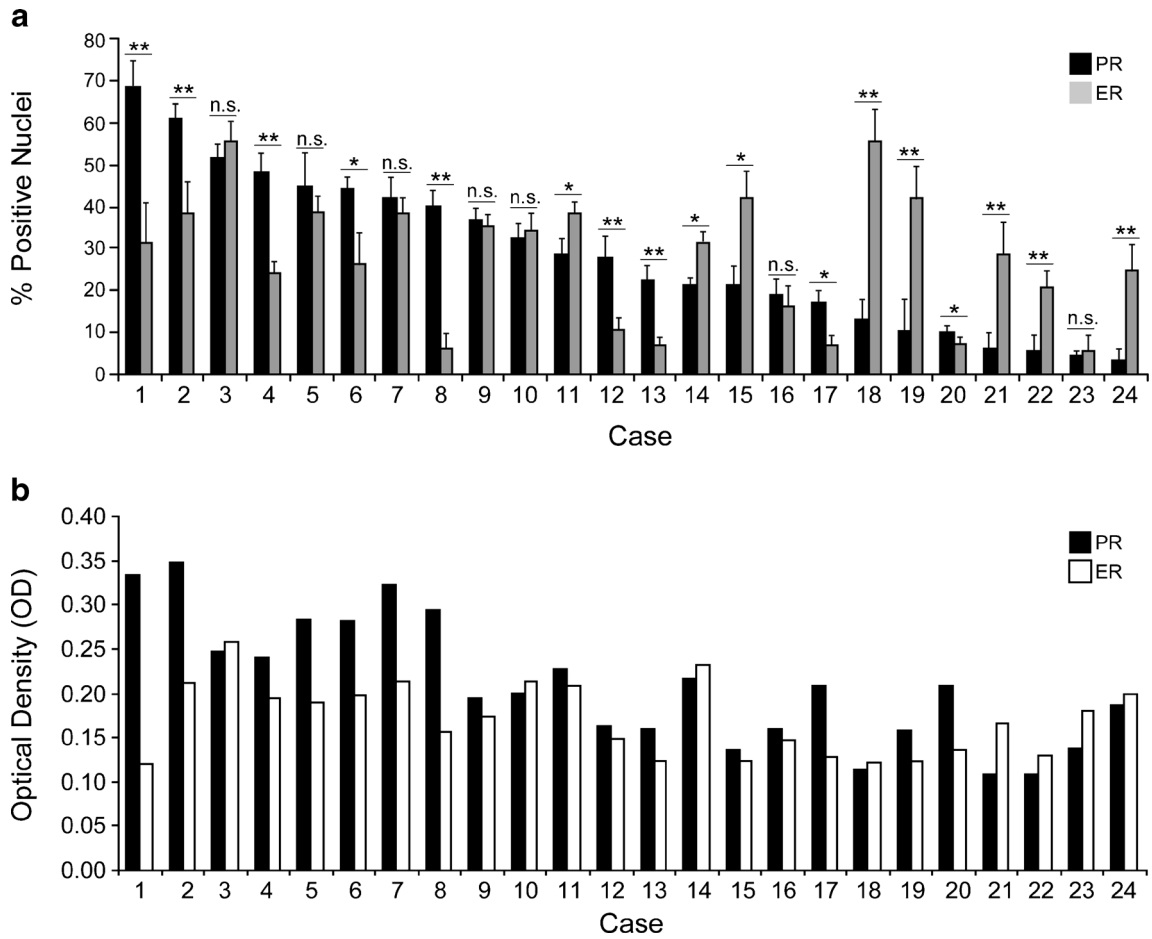

Fig. 4 Quantitative image analysis of PR and ER expression in explanted cases of LAM. **a** Percentage of total PR-positive and ER-positive nuclei per case. Data represent mean±SD. $^{**}p<0.01$, $^{*}p<0.05$ and *n.s.*, non-significant, by one-way ANOVA. **b** Percentage of total stained PR-positive and ER-positive nuclei absorption is shown in log scale by *optical density* (*OD*) [OD=log10 (240/average positive intensity)]

PR and ER expression. These cases had in common the presence of abnormal lung function consistent with a more advanced stage of disease, (in the biopsy material) or definite pulmonary insufficiency, in the case of explanted cases of LAM. However, the single biopsy case with more ER than PR expression had normal lung function, and nine cases of explanted cases of LAM had more PR than ER; therefore, PR/ER ratio per se does not seem to be associated with the clinical severity of the disease.

In nine cases (21 % of total cases) including one biopsy (5 % of all biopsies) and eight explanted cases of LAM specimens (33 % of all explanted cases of LAM specimens), ER expression predominated over PR in a statistically significant manner. The difference in PR positivity between the explanted cases of LAM and the diagnostic biopsy material

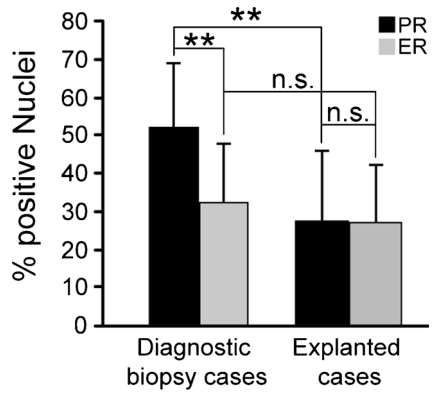

Fig. 5 Comparison of total PR-positive nuclei between biopsy ($n=20$) and explanted ($n=24$) LAM cases. Data represent mean±SD. $^{**}p<0.01$, $^{*}p<0.05$ and *n.s.*, non-significant, by one-way ANOVA

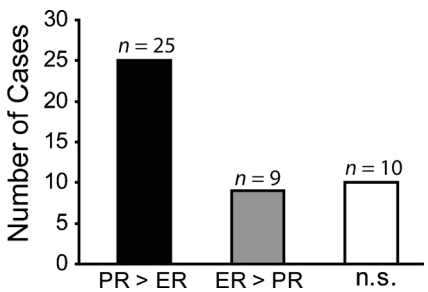

Fig. 6 Number of cases statistically positive for PR over ER, statistically positive for ER over PR, and with no statistically significant differences between the two receptors (*n.s.*)

was due to relatively fewer PR-positive cells in explanted cases of LAM specimens, rather than to an increase in ER expression, which was similar in biopsies and explanted cases of LAM. These findings may be taken to suggest that the lower expression of PR in explanted cases of LAM is a reflection of the natural progression of the disease, but this cannot be taken as a confirmed fact. First, because the biopsy and the explanted cases of LAM samples were obtained from different patients, as opposed to samples from the same person obtained at different times. Second, because the performance of open lung biopsies requires a significantly shorter surgery time than the removal of whole lung specimens; therefore, the level of receptor degradation should potentially be higher in the latter (explaining the lower PR detection in explanted cases of LAM lung specimens). This second caveat is not supported by the fact that ER degradation in cultured cells is faster than that of PR (5 versus 21 h, [63, 64]), implying that we should mainly see lower ER expression in the explanted lungs than in the biopsies.

Two TSC-LAM cases were included in our study, one was a biopsy case and the other one was an explanted case (Figs. 1a and 4a). The biopsy showed a high PR/ER ratio; however, the explanted case showed higher ER than PR. Although limited in number, these findings suggest TSC-LAM behaves as S-LAM in terms of ER and PR expression.

LAM is unusual in its high PR/ER ratio; other more common female neoplasms, such as endometrial carcinoma [48] and some breast cancer [51, 52], show a definite prevalence of ER, when they are not negative for both receptors. The high PR expression may have clinical repercussions for the treatment of LAM. So far, however, the role of progesterone in LAM has not been explored, nor have treatments aimed at reducing its effect been tested. Unlike in LAM, the role of progesterone in the development of breast and endometrial cancer has been extensively studied. It is well established that progesterone plays a protective role in endometrial cancer [53–55]. The fact the progestins did not help LAM patients, however, suggests that it does not play such a role in LAM. A large clinical trial with hormonal therapy found that progestins instigated breast cancer in post-menopausal women [56], which highlights the proliferation stimulating role of progestins in breast cancer unlike in endometrial cancer [56–59]. Studies have shown that through PR, progestins influence the activation of pro-proliferative proteins such as cyclin D_1, MAPK, and PI3K/Akt/mTOR pathway in breast cancer cells [60–64], and it might have a similar effect in LAM.

In conclusion, our study shows that PR expression is significantly higher than ER expression predominantly in biopsy cases of pulmonary LAM in terms of percentage of positive nuclei and intensity of the immunoreactivity. This finding suggests that PR may play a more prominent role in LAM than previously considered, and that PR antagonists might be considered a treatment option.

Conflict of Interest The authors declare no conflicts of interest.

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