Antibody α PEP13h Reacts With Lymphangioleiomyomatosis Cells in Lung Nodules

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BACKGROUND: Lymphangioleiomyomatosis (LAM) is characterized by the proliferation in the lung, axial lymphatics (eg, lymphangioleiomyomas), and kidney (eg, angiomyolipomas) of abnormal smooth muscle-like LAM cells, which express melanoma antigens such as Pmel17/gp100 and have dysfunctional tumor suppressor tuberous sclerosis complex (TSC) genes *TSC2* or *TSC1*. Histopathologic diagnosis of LAM in lung specimens is based on identification of the Pmel17 protein with the monoclonal antibody HMB-45.

METHODS: We compared the sensitivity of HMB-45 to that of antipeptide antibody α PEP13h, which reacts with a C-terminal peptide of Pmel17. LAM lung nodules were laser-capture microdissected to identify proteins by Western blotting.

RESULTS: HMB-45 recognized approximately 25% of LAM cells within the LAM lung nodules, whereas α PEP13h identified > 82% of LAM cells within these structures in approximately 90% of patients. Whereas HMB-45 reacted with epithelioid but not with spindle-shaped LAM cells, α PEP13h identified both spindle-shaped and epithelioid LAM cells, providing greater sensitivity for detection of all types of LAM cells. HMB-45 recognized Pmel17 in premelanosomal organelles; α PEP13h recognized proteins in the cytoplasm as well as in premelanosomal organelles. Both antibodies recognized a Pmel17 variant of approximately 50 kDa.

CONCLUSIONS: Based on its sensitivity and specificity, α PEP13h may be useful in the diagnosis of LAM and more sensitive than HMB-45.

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ABBREVIATIONS: LAM = lymphangioleiomyomatosis; mTOR = mammalian target of rapamycin; TSC = tuberous sclerosis complex

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Lymphangioleiomyomatosis (LAM) is a rare lung disease found predominantly in women and characterized by abnormal proliferation of smooth muscle-like cells (LAM cells) in the lung and other involved organs.¹⁻³ Although LAM cells possess mutations in the tuberous sclerosis complex (TSC) genes *TSC1* or *TSC2*, which encode hamartin and tuberin, respectively,⁴⁻⁶ lack of *TSC2* exonic mutations in cells from LAM lung nodules has been reported.⁷ Expression of tuberin may be negatively regulated by DNA methylation.⁸

Dysfunctional hamartin or tuberin leads to increased activity of the mammalian target of rapamycin (mTOR), resulting in increased cell size and proliferation.⁹⁻¹¹ As a result, mTOR has been targeted in clinical trials with rapamycin (sirolimus) or related rapalogs.¹²⁻¹⁵

Histopathologically, LAM cells have been identified by the presence of Pmel17, a protein that reacts with the monoclonal antibody HMB-45, which was generated against a melanoma antigen.¹⁶⁻¹⁹ Pmel17 (also called silver protein and ME20) is a splice variant of the *Pmel17* gene product, which is involved in pigmentation and required for the normal formation of stage I and II melanosomes and melanin deposition.²⁰⁻²² The *Pmel17* gene comprises 11 exons encoding a protein of approximately 71 kDa, but due to glycosylation, the protein has a molecular mass of around 100 kDa. Splicing of the last exon of *Pmel17* generates gp100.²³ Detection of Pmel17 with HMB-45 in biopsy specimens is generally used for the histopathologic diagnosis of LAM. This antibody identifies LAM cells in explanted lungs as well as in sections from open-lung and transbronchial biopsy samples and other extrapulmonary LAM lesions in the lymphatics and kidneys.¹⁶ LAM lung nodules comprise different cell types. The HMB-45 antibody mainly recognizes the epithelioid cell type but not the more spindle-shaped, proliferative LAM cells.¹⁷

Pathologic sections (ie, biopsy specimens and explanted tissues) of patients with LAM could be described as proliferative (nodular) or cystic. The degree of involvement with lung lesions has helped to establish a histologic score, which is useful to define the stage of disease.^{19,24}

Most information on LAM pathologic sections describes nodular structures, which contain two main types of LAM cells: spindle-shaped and epithelioid. Spindle-shaped cells are centrally located, proliferative LAM cells characterized by the presence of proliferating cell nuclear antigen and Ki67, a protein regulated during the cell cycle, and membrane type-1 matrix metalloproteinase.^{17,25,26} In contrast, epithelioid cells are found at the periphery of the LAM lung nodules and have been identified as those that react to the monoclonal antibody HMB-45 and antiestrogen and antiprogesterone receptor antibodies.²⁵ Recently, it has been reported that most LAM cells contain progesterone receptor.²⁷ HMB-45 reacts with the Pmel17 gene product gp100 found in LAM cells.¹⁷ The LAM lung nodules are surrounded by hyperplastic type 2 pneumocytes.²⁸ LAM nodules also contain mast cells²⁹ and are infiltrated by lymphatic vessels.³⁰

Because only a minority of LAM cells react with the HMB-45 antibody, HMB-45 is not always helpful in diagnosis, especially with small specimens (eg, from transbronchial biopsy). HMB-45 recognizes a region within the central polycystic kidney disease domain of Pmel17.^{21,22} Another antibody of interest, α PEP13h, recognizes an amino acid sequence in the C-terminal portion of Pmel17³¹ and appears, as in our preliminary studies, to identify a different spectrum of LAM cells in lung nodules. To address the question of LAM cell recognition, we first investigated the presence of Pmel17 in LAM cells and then compared HMB-45 with α PEP13h reactivity. Because the intracellular processing and sorting of Pmel17 is complex and has been extensively studied, we looked for Pmel17 variants^{17,32} in LAM cells. Next, we investigated the intracellular structures of LAM cells, which may contain these proteins or their isoforms.

In contrast to HMB-45, which recognizes Pmel17 in melanosomal structures in a small fraction of smooth muscle actin-positive cells, we show that the α PEP13h antibody recognizes Pmel17 in the cytoplasm and pre-melanosomes of > 82% of LAM cells in 90% of patients with LAM. α PEP13h may help in the diagnosis of LAM and other perivascular epithelioid cell neoplasms.

Materials and Methods

Patients

The study group comprised 22 women (mean age $\pm$ SD, 39.3 $\pm$ 8.6 years) in whom the diagnosis of pulmonary LAM was based on previously defined clinical and pathologic criteria^{18,33-35} and whose tissues were available for analysis. One of these patients had clinical evidence of TSC. The study was approved by the National Heart, Lung, and Blood Institute Institutional Review Board (protocols #95-H-0186 and 95-H-0100).

Patients provided written informed consent. To test our hypothesis, tissues for transmission electron microscopy and for rapid freezing were obtained from 10 patients with LAM (mean age, 37.6 $\pm$ 12.2 years) in whom sufficient lung tissue was available for the immunohistochemical analyses.

Cells

Cultured human pigmented MNT-1 melanocytes (a gift from Prof PierGiorgio Natali, MD, Regina Elena Institute, Rome, Italy) and Malme-3M

melanoma cells (ATCC) were grown as previously described.¹⁷ Cultures of cells from explanted lungs also were as described previously.³⁶

Histopathology

Paraffin sections of formalin-fixed tissues were stained with hematoxylin-eosin and by the Fontana-Masson method. Frozen sections of unfixed lungs from 10 patients were fixed with either cold acetone or cold 4% p-formaldehyde for 15 min. For reactions with a single antibody, paraffin and frozen sections were immunostained by the peroxidase method as described previously.¹⁹ For the use of two antibodies, tissue sections were stained by the double indirect immunofluorescence method (using fluorescein isothiocyanate- and Texas Red-conjugated secondary antibodies) as previously described.¹⁹ In these preparations, immunoreactive cells were classified into three categories according to whether they showed green, red, or yellow fluorescence. Nuclei were counterstained with DAPI (4',6-diamidino-2-phenylindole) (blue fluorescence). Vascular and bronchial smooth muscle cells, which were present in LAM lung sections, were not reactive with HMB-45 or α PEP13h. α PEP13h was raised against the polypeptide 647-CGLGENSPLLSGQV-661.^{20,21} HMB-45 was raised against an extract of pigmented melanoma²³; the region identified by this antibody has been mapped to a central portion of the Pmel17 protein between amino acids 315 and 444.²² All

readings were done independently by two pathologists in a blinded manner.

Electron Microscopy

Lung samples were left unfixed for frozen sections. Tissue samples were fixed in 2% glutaraldehyde for electron microscopy as described previously.^{37,38}

Poly A-RNA Isolation and Reverse Transcriptase Polymerase Chain Reaction Assay

mRNA was extracted from LAM tissue samples using an Oligotex Direct mRNA Kit (QIAGEN). Processing details are provided in e-Appendix 1.

Sample Preparation and Immunoblot Analysis

Proteins were extracted from frozen tissue sections of LAM lungs using laser-capture microdissection. Processing details are provided in e-Appendix 1.

Statistical Analysis

All values are presented as mean $\pm$ SD. The Kruskal-Wallis test was used to analyze nonparametric data. Differences were considered significant at $P < .05$.

Results

We investigated the presence of Pmel17 in LAM lung nodules. Both *Pmel17* and *gp100* mRNAs were present in LAM lung nodules as well as in MNT-1 melanoma cells (Fig 1A). Only a subpopulation of LAM cells, however, reacted to the HMB-45 antibody,¹⁷ which recognized a region in the processed repeat domain of Pmel17 and gp100. We assessed the status of melanosomes in LAM cells by electron microscopy (Fig 1B). As reported previously, in general, there were very few well-developed early melanosomes in LAM cells. Noticeably, remnants of Pmel17-derived amyloid fibrils were found inside either multivesicular structures or disrupted premelanosomes, supporting the hypothesis that instability exists as a result of a truncated melanosome maturation process (Fig 1B). Thus, it is possible that the stability and conformation of Pmel17 amyloid fibers is compromised due to exposure to uncontrolled and nonmelanosomal environments.

We next focused on detection of Pmel17 using both HMB-45 and α PEP13h, an antibody that recognizes the C-terminal region of Pmel17.³¹ As shown in Figure 1C, immunoblotting assays using proteins extracted from laser-capture microdissected LAM nodules revealed that HMB-45 detected a weak single band at approximately 50 kDa in only some LAM lung specimens. This band may correspond to one of the processed forms of Pmel17 seen in control samples from melanoma cells (Fig 1C, middle). In contrast, α PEP13h detected both full-length Pmel17 (approximately 98 kDa) (Fig 1C, *) and the C-terminal region (approximately 22 kDa) (Fig 1C, <, bottom). These data suggest that the

C-terminal region is more readily detected than the repeat domain of Pmel17 in LAM tissues, and that α PEP13h is a much more inclusive antibody because it detects full-length and processed protein products.

In general, LAM lung specimens reacted with α PEP13h and HMB-45 antibodies. However, a larger number of LAM cells reacted with α PEP13h ($82.23\% \pm 10.34\%$) than with HMB-45 ($25.56\% \pm 6.12\%$, $P < .05$) (Fig 2). The study group comprised 22 women (age, 39.3 ± 8.6 years) in whom the diagnosis of pulmonary LAM was made based on previously defined clinical and pathologic criteria. In both paraffin and frozen sections, a granular cytoplasmic reaction of premelanosomes was observed with HMB-45 (Figs 2A, 2B), whereas α PEP13h reactivity tended to be diffusely localized within the cytoplasm as well as in the premelanosomes (Figs 2C, 2D). Immunoreactivity of α PEP13h and HMB-45 was observed in the cytoplasm of epithelioid cells (Figs 2B, 2D); however, as noted previously, a much greater proportion of LAM cells, both spindle-shaped and epithelioid, were positive for α PEP13h than for HMB-45.

It has also been reported that other melanosomal proteins, including MART-1 and Melan A, are present in a minority of LAM cells.³⁹ Interestingly, the lysosomal and membrane-associated protein tetraspanin CD63, or LAMP3,²⁶ is highly expressed in LAM cells. Although other melanosomal proteins have been investigated in LAM cells, the results appear more variable for proteins such as tyrosinase and tyrosinase-associated protein.^{39,40} In addition, antimelanosomal-specific antibodies, or PNL2,²⁶ react with a large number of LAM lung nodules. However, LAM cells are more definitively

Figure 1 – Expression of Pmel17 in LAM nodules and melanoma cells. A, Detection of Pmel17 (NM_006928) and gp100. Gp100 is generated by an in-frame deletion of 21 bp in exon 10 of Pmel17 mRNA. Representative reverse transcriptase-polymerase chain reaction assay of the Pmel17/ gp100 gene. Twenty micrograms mRNA were reverse transcribed using primers to amplify either Pmel17- or gp100-specific regions of the gene. Poly-A mRNA was obtained from seven LAM cases and a melanoma cell line (MNT-1). We did not run a sample in the middle lane. B, Electron micrograph. Epithelioid LAM cells have large nuclei, well-developed endoplasmic reticula, Golgi complexes, glycogen particles, and stage II melanosomes (left, arrow) (Kajikawa stain, original magnification $\times 5,000$). The inset shows a higher-magnification view of the stage II melanosomes, which contain lamellate inclusions. Several multivesicular

identified for their reactivity to HMB-45 and now α PEP13h.

The present results suggest that the α PEP13h antibody detected Pmel17 better than HMB-45 in LAM cells. To confirm these findings, the dual immunofluorescence method was performed combining α PEP13h or HMB-45 with anti-smooth muscle actin antibodies. Confocal microscopic images were taken of frozen sections of LAM lung tissue following dual labeling. Figures 2E and 2F show colocalization of the two reactions in yellow, and nuclei showed blue fluorescence due to counterstaining with DAPI. A population of cells was reactive only with α PEP13h (Figs 2E, 2F). Of note, most of the HMB-45-positive cells were not strongly reactive to anti-smooth muscle actin antibodies. The variability on staining is probably due to the different phenotypes of LAM cells and is consistent with the epithelioid cell appearing more differentiated. Cells cultured from explanted LAM lungs were also reactive to α PEP13h (Figs 2G, 2H), and interestingly, a cross-sectional view of the immunostaining allowed us to visualize the colocalization and internal distribution of the proteins. The data suggest that the proteins are processed intracellularly and are probably present as a soluble form in the cytosol and in premelanosomal structures.

Discussion

The present results confirm a central role for Pmel17/gp100 as a marker in the diagnosis of LAM based on immunodetection of the processed forms of this protein with HMB-45 and α PEP13h. Although LAM cells are genetically characterized by alteration of the tumor

bodies containing remnants of fibers were observed in some LAM cells (right top and bottom, arrows) (Kajikawa stain, original magnification $\times 15,000$). C, Schematic representation of Pmel17 protein showing the regions that react with HMB-45 and α PEP13h antibodies (top). Squares indicate the known domains in Pmel17, top numbers indicate the amino acid location of N-terminal glycosylation sites, and bottom numbers indicate the amino acid start and end positions for the different domains. Small graphics over the RPT domain indicate multiple O-linked glycosylation sites. Proteins from cells obtained by laser-capture microdissection were immunoblotted with HMB-45 (middle) and α PEP13h (bottom) antibodies. All lanes were loaded equally, and no lanes were left blank. The HMB-45 antibody reacted with a band corresponding to approximately 50 kDa in Malme-3M and MNT-1 cells but not in the NL control sample. The HMB-45 antibody reacted mainly with a 51-kDa protein band in several LAM samples. The α PEP13h antibody recognized a band of approximately 50 kDa in all LAM tissues. MNT-1 cells and Malme-3M cells showed a major band of approximately 90 kDa (*). An approximately 22-kDa band (<) was detected with the antibody α PEP13h. No band was observed in an NL sample. bp = base pair; CS = furin-cleaved site; CTD = C-terminal domain; KRG = kringle-like domain; L = DNA ladder; LAM = lymphangioleiomyomatosis; NL = normal lung; NTD = N-terminal domain; PKD = polycystic kidney disease-like domain; RPT = repeat domain; SD = signal domain; TM = transmembrane domain.

Figure 2 – Immunohistopathology of *Pmel17* gene products in LAM nodules. A, HMB-45 reactivity was localized mainly in scattered cells within LAM nodules. B, A granular cytoplasmic reactivity was observed in epithelioid LAM cells. C and D, Reactivity to anti- α PEP13h was found distributed in the majority of LAM cells (C) with diffuse cytoplasmic reactivity, which was accentuated in the perinuclear regions of LAM cells (D). E and F, Dual staining for α PEP13h (green) (E) or HMB-45 (green) (F) with smooth muscle actin (red) antibody in LAM cells (arrows). G and H, Cultured cells from explanted lungs were reactive to α PEP13h (green) and calponin (red), a smooth muscle-specific protein. H shows a cross section of cells in G to demonstrate the intracellular staining of LAM cells. Nuclei were counterstained with DAPI (4',6-diamidino-2-phenylindole) (blue fluorescence). (Magnifications, A and B, $\times 100$; C, D, F, G $\times 630$; E and H $\times 480$.) See Figure 1 legend for expansion of abbreviation.

suppressor genes *TSC2*, *TSC1*, or both⁴ leading to hyperactivation of mTOR and to deregulated cell proliferation, the relationship of *Pmel17* and *TSC2/TSC1* gene products remains to be determined. Currently, histopathologic diagnosis of LAM is based on the presence of *Pmel17* in LAM lung nodules as detected by reactivity to HMB-45.¹⁶ A greater proportion of LAM cells

were identified with the antibody α PEP13h than with HMB-45, which is currently used for diagnosis.^{2,18} α PEP13h recognized the melanosomal as well as the cytosolic fraction of *Pmel17* in α -smooth muscle-positive cells.

Pmel17 is a structural melanosomal protein that participates in melanosomal architecture, fibrillar formation, and melanin deposition.^{41–43} The predicted size of the *Pmel17*/gp100 core protein is approximately 70 kDa; however, due to glycosylation, *Pmel17* is seen by sodium dodecyl sulfate-polyacrylamide gel electrophoresis as an approximately 100-kDa protein.²³ With proteins obtained from LAM nodules, HMB-45 and α PEP13h antibodies recognized protein bands of around 50, 23, and 98 kDa (Fig 1), which could result from alternative processing of the mRNA or protein (eg, splicing, glycosylation, proteolysis). In fact, *Pmel17*, following insertion into melanosomal membranes, is initially sorted to stage I melanosomes where it can be recognized by α PEP13h but not by HMB-45 (data not shown).²¹ Once *Pmel17* is translocated to melanosomal membranes, it is cleaved and becomes part of the fibrillar matrix of stage II melanosomes. In this location, *Pmel17* is recognizable by HMB-45. We have previously shown that HMB-45 reactivity is located at the perinuclear region.³⁶ Thus, although LAM cells expressed proteins encoded by the *Pmel17* gene, the gene or its protein products may undergo processing in LAM cells, which may alter antibody reactivity. Interestingly, we were able to detect an approximately 22-kDa band, which resulted from further posttranslational processing of the *Pmel17* in melanoma cells but had not been previously reported in LAM lung nodules.²¹ Pulse-chase experiments have shown that the 22-kDa protein product is a fragment of *Pmel17*.²¹ Thus, the molecular weights of the proteins detected in laser-capture microdissected LAM nodules could be partly explained by alternative splicing and posttranslational modification, which differs from that detected in melanocytic cells,²¹ resulting in the identification of a protein of around 50 kDa. The results with HMB-45 are consistent with those found previously with protein extracts of LAM lungs.¹⁷

The reactivity of the α PEP13h was mainly cytosolic, suggesting a mislocalization of *Pmel17*; incomplete glycosylation; and disrupted formation of structures, disassembly of fibrillar structures, or both within LAM cells. Incomplete glycosylation and processing of *Pmel17* could lead to abnormal transport of these proteins from the Golgi complex to the melanosomes. Because *Pmel17* fibers, due to their pH sensitivity, depend on the structural

stability of melanosomes, we infer that those features are altered in LAM cells and limit detection of Pmel17 by HMB-45. Similarly, disassembly of Pmel17 depositions regulated by changes of intracellular pH could explain the cytosolic localization of the protein (Fig 2). Of note, HMB-45 reacts with epithelioid cells, which are the minority of cells within a LAM lung nodule, but not with the proliferative cells.¹⁷ In contrast, α PEP13h reacted with both epithelioid and spindle-shaped cells. Furthermore, it was more common to detect reactivity to α PEP13h than to HMB-45. Although we could not determine quantitatively the number of cells negative for HMB-45 and positive for α PEP13, we observed in serial sections that at least 70% of cells reactive to α PEP13 were not identified with HMB-45 (Figs 2E, 2F).

We previously reported the reactivity of cells cultured from LAM lungs toward HMB-45. We found that few cells reacted with this antibody. However, when detected,

most of the reactivity of these cells was localized in intracellular structures closely associated with the cell nucleus.³⁶ The intracellular distribution of α PEP13h reactivity in cultured cells from LAM lungs seems similar to the distribution of cells found in the LAM lung nodules (Fig 2), and cultured LAM cells also possess a smooth muscle-like phenotype. Despite the molecular insights into LAM cells, we lack understanding of the relationship between alterations of the *TSC* genes and the melanocytic phenotype of LAM cells.

The distribution of the Pmel17/gp100 family of proteins may facilitate the recognition of LAM cells. Because more LAM cells are reactive with α PEP13h than with HMB-45, α PEP13h may be a preferred diagnostic antibody for LAM. This reactivity can be used as a marker for LAM cells in pathologic specimens obtained from open-lung, transbronchial biopsies and other pathologic specimens, especially when tissue is limiting.^{16,18}

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Additional information: The e-Appendix can be found in the Supplemental Materials section of the online article.

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