**TSC2 Loss in Lymphangioleiomyomatosis Cells Correlated with Expression of CD44v6, a Molecular Determinant of Metastasis**

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**Abstract**

Lymphangioleiomyomatosis (LAM), a rare multisystem disease found primarily in women of childbearing age, is characterized by the proliferation of abnormal smooth muscle–like cells, LAM cells, that form nodules in the pulmonary interstitium. Proliferation of LAM cells results, in part, from dysfunction in tuberous sclerosis complex (TSC) genes TSC1 (hamartin) and/or TSC2 (tuberin). Identification of LAM cells in donor lungs, their isolation from blood, and their presence in urine, chylous ascites, and pleural effusions are consistent with their ability to metastasize. Here, we investigated the presence on LAM cells of the hyaluronic acid receptor CD44 and its splice variants associated with metastasis. The heterogeneous populations of cells grown from lungs of 12 LAM patients contain cells expressing mRNA for the variant CD44v6. Histologically, CD44v6 was present in LAM lung nodules, but not in normal vascular smooth muscle cells. CD44v6-positive sorted cells showed loss of heterozygosity at the TSC2 locus; binding of CD44v6 antibody resulted in loss of cell viability. Levels of CD44 were higher in cultured Eker rat (Tsc2–/–) cells than in Tsc2+/+ cells, but unlike human LAM cells, the Tsc2–/– Eker rat cells did not contain CD44v6 splice variant mRNA. CD44 splicing and signaling is regulated by osteopontin. Plasma from LAM patients contained higher concentrations of osteopontin than plasma of healthy, age-, and sex-matched volunteers (P = 0.00003) and may be a biomarker for LAM. The cell surface receptor CD44 and its splice variant CD44v6 may contribute to the metastatic potential of LAM cells. [Cancer Res 2007;67(21):10573–81]

**Introduction**

Lymphangioleiomyomatosis (LAM), a rare multisystem disease found primarily in women of childbearing age, presents in a sporadic manner or in association with tuberous sclerosis complex (TSC), an inherited disorder. LAM is characterized by the aberrant proliferation of smooth muscle–like cells (LAM cells) that form nodules in the pulmonary interstitium (1, 2). In addition, LAM or LAM/TSC may be associated with renal angiomylipomas and infiltration of the axial lymphatics. Patients presenting with TSC may exhibit typical skin lesions, including angiofibromas and periungual fibromas (3).

The LAM lesions in lungs of patients with sporadic presentation of the disease possess mutations in the TSC2 gene, which encodes tuberin (4). TSC protein abnormalities in LAM may lead to dysregulation of the mammalian target of rapamycin, with increased p70 S6 kinase activity (5, 6). In addition to abnormalities in TSC, LAM cells within the nodular lung structures contain proteins that react with antibodies against the Pmel17 gene product, gp100 (HMB-45 immunoreactivity) and MART-1 (1, 7). The LAM cells also exhibit a smooth muscle phenotype and contain immunoreactive smooth muscle actin (SMA). The relationship of the smooth muscle–like and melanocytic phenotypes to mutations in the TSC genes is not clear.

The multisystem manifestations of LAM have been correlated with a metastatic dissemination of the LAM cells (4, 8, 9), which were identified in donor lungs transplanted to LAM patients (10, 11). Furthermore, LAM cells have been detected in blood, chyle, and urine (8). Thus, metastasis seems to be a mechanism by which LAM cells are disseminated but the molecular determinants are unknown.

CD44 is a class I transmembrane glycoprotein encoded by 21 exons present in chromosome 11, and specific splice isoforms of this molecule have been identified as molecular determinants of metastasis (12–14). The standard form (CD44s) encoded by exons 1 to 5 and 6 to 10 is found ubiquitously with a predicted molecular weight of ~37 kDa; however, the proteins identified by monoclonal antibodies range in size from 80 to 200 kDa due to glycosylation variants. The production of CD44 splice isoforms are regulated mainly by signaling mechanisms involving the Ras–mitogen-activated protein kinase pathway (12, 15). Isoforms generated by splicing using the 10 additional exons v1 to v10 result in an insertion of a segment within the extracellular portion of the molecule.

The CD44 extracellular domain is responsible for binding of hyaluronic acid, E-selectin, osteopontin, hepatocyte growth factor, and Fas ligand (12, 16, 17). In addition, the extracellular domain of CD44 binds metalloproteases, including MMP7 and MMP9, which cleave CD44s as well as variant 6 (CD44v6; ref. 18). CD44v6 has been associated with tumorigenicity and is involved in homing during metastasis (13).

CD44 splicing is regulated, in part, by growth factors such as osteopontin and hepatocyte growth factor (19, 20). Osteopontin is chemokine-like molecule with multiple domains including an arginine-glycine-aspartate (RGD) domain, which promotes binding to integrins, and a CD44-binding domain. It also contains a thrombin cleavage site and can be a substrate for MMP7 (21, 22). Multiple cell types, including smooth muscle cells, express this adhesive glycoprotein. Interestingly, osteopontin binds CD44v6 and CD44v3 (23, 24). Thus, CD44 and its splice variants are involved in multiple cellular processes required for metastasis, and osteopontin seems to be one of the main regulators of CD44 function and splicing. Lungs are common sites of metastases for breast cancer.

**Note:** Supplementary data for this article are available at Cancer Research Online (http://cancerres.aacrjournals.org/).

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and melanoma among other malignancies (25, 26), in which the hyaluronic acid receptor CD44 plays a role (12). The splice variant CD44v6 has been associated with and was shown to function in multiple metastatic tumors (27). Because LAM cells seem to have metastatic potential, we asked whether CD44 and its splice variants are expressed in LAM cells. The variant CD44v6 was detected on cultured LAM cells from explanted lung with loss of heterozygosity (LOH) for TSC2. LAM cells within the lung nodules, but not normal vascular cells, expressed CD44v6 on the exterior surface, which may contribute to their metastatic potential and be a potential therapeutic target.

Materials and Methods

Patient population. The study group comprised patients (age 34–54 years) in whom the diagnosis of LAM was made on the basis of clinical, radiologic, and histopathologic findings. Tissue was obtained at the time of lung transplantation or elective skin biopsy. The patients consented under a protocol (95-H-0186) approved by the National Heart, Lung, and Blood Institutional Review Board.

Isolation of cells from LAM lung. To culture cells from explanted lungs of LAM patients, lung sections (1–3 mm) obtained at the time of transplant were placed on plastic dishes. Cells were grown in mesenchymal cell culture medium (Cambrex) for 1 week. Mesenchymal cell medium contains 0.05 unit/mL of penicillin, 0.05 µg/mL of streptomycin, and 4 mmol/L glutamine supplemented with 10% fetal bovine serum, which promotes the growth of mesenchymal stem cells as determined by the potential of the grown cells to differentiate to adipocytes, chondrocytes, and osteoclasts (28). When small colonies of cells were formed, the tissue sections were removed and the cells were allowed to grow for 3 to 7 days before trypsinization and transfer to new plastic dishes. This procedure seems to select a mixture of cells that include those with LOH for TSC2.

Fluorescent in situ hybridization. Cells grown in LabTek chambers were washed with Dulbecco’s PBS (DBPBS) containing 1 mmol/L CaCl₂ and 1 mmol/L MgCl₂. Cells were fixed in methanol/glacial acetic acid (3:1) at −20°C and incubated for 30 min at −20°C. Slides were air dried and stored at −20°C until use. A 199-kb BAC clone encompassing the TSC1 gene (RP11-295G24) was purchased from Research Genetics and CDEB9, a 32-kb cosmid clone spanning the 5′ untranslated region through exon 13 of the TSC2 gene, was generously provided by Dr. David Kwiatkowski (Brigham and Women’s Hospital, Boston, MA). Probes were labeled directly using Spectrum Green (TSC1) or Spectrum Orange (TSC2; Vysis). Nuclei were stained with 4′,6-diamidino-2-phenylindole (DAPI). Images obtained using a BX40 fluorescence microscope (Olympus) fitted with an Orca ER digital charge-coupled device camera (Hamamatsu) were merged and analyzed using OPENLAB 3.0 software (Improvision). On average, ~20% of cells from initial cultures showed allelic deletion for TSC2.

Immunohistochemistry. Immediately after removal of lung tissue at autopsy. The tissues were dehydrated, embedded in paraffin, and sectioned to a thickness of 5 µm. To evaluate the localization and amount of LAM cellswithinthelungnodules, butnotnormal lung tissue was obtained. Lung nodules were found in all women (age range 34–54 years) in whom the diagnosis of LAM was made on the basis of clinical, radiologic, and histopathologic findings. Lung nodules were found in all women (age range 34–54 years) in whom the diagnosis of LAM was made on the basis of clinical, radiologic, and histopathologic findings. Lung nodules were from one biopsy at the time of the patient’s initial diagnostic evaluation, in eight patients from explanted lung, and in one patient at autopsy. The tissues were dehydrated, embedded in paraffin, and sectioned to a thickness of 5 µm. To evaluate the localization and amount of immunoreactive CD44 and CD44v6, sections were immunostained by the immunohistochemical staining and laser scanning confocal fluorescence microscopy. Immunohistochemistry was used to evaluate colocalization of immunoreactivity for CD44v6 and TSC2. Dual staining for CD44v6 and other tumor markers (19, 20) was carried out on lung tissue sections from LAM patients. Sections were incubated overnight at 4°C with a mixture of the two primary antibodies (diluted 1:20 and 1:10, respectively), and then for 1 h with FITC-labeled goat antibody against mouse IgG (1:100) and Texas red–labeled rabbit antibody against rabbit IgG (1:100; Vector Laboratories). After washing with PBS, sections were incubated with DAPI (Sigma Chemical) for 15 min and inspected with a confocal microscope (model TCS4D/DMIRB8; Leica), equipped with argon and krypton laser sources.

Immunostaining of cultured cells. Cells grown as described were transfected into LabTek II slides, incubated overnight, and washed with PBS containing 1 mmol/L each of CaCl₂ and MgCl₂. Then, cells were fixed with 4% paraformaldehyde (Electron Microscopy, Inc.) and permeabilized with 0.1% Triton X-100 for 4 min at room temperature, followed by incubation with DPBS containing 3% bovine serum albumin and 5% goat serum for 1 h at room temperature. Cells were washed with DPBS, incubated with the indicated antibody at 4°C for ~12 h, washed again with DPBS, and incubated with secondary antibodies labeled with the indicated fluorophore for 1 h at room temperature. After washing, the slides were covered with Vecastain (Vector Laboratories) with or without DAPI and sealed with a coverslip.

Quantitative analysis of cell fluorescence by confocal microscopy. Quantitative data images were acquired using identical settings of the instrument that avoid saturation of the brightest pixels. Series of 12-bit images along the z-axis were obtained throughout the cells with a x40, 1.3-numerical-aperture Plan-Apochromat oil immersion objective. Analysis was done using Imaris 5.0.2 software package (Bitplane AG). The integrated sum of total fluorescence through all optical sections in the three-dimensional series is calculated and compared between EEF– and EEF+/− cells. Reverse transcription-PCR of CD44 mRNA. mRNA was extracted from cells using the RNeasy mini kit from Qiagen; cDNA was prepared with the Superscript First Strand Synthesis System for reverse transcription-PCR (RT-PCR; Invitrogen); and PCR was done using primers described by Hudson et al. (29). As controls, PCR was done with actin- and/or glyceraldehyde-3-phosphate dehydrogenase-specific primers (Biosource International).

Fluorescence-activated cell sorting. Confluent cells in 100-mm dishes were washed with 10 mL PBS without calcium and magnesium and incubated with 1 mL 25 mmol/L EDTA and 0.25% trypsin. When cell detachment was observed microscopically, 10 mL of complete medium were added. Cells (~2 × 10⁶) collected by centrifugation at 500 × g were washed once with 10 mL DBPBS, suspended in DPBS, and incubated for 30 min at room temperature with antibodies CD44-R-phycocerythrin (clone F10-44-2, Biosource International) and CD44v6-fluorescein (clone VFF-7, Biosource International) for human cells. Anti-CD44-fluorescein (clone OX-49) was used to detect expression of CD44 in rat cells, and anti-human CD9 (clone MM2/57) labeled with either RPE or fluorescein was also used to help in the identification of LAM cells.

7-Amino-actinomycin D (7AAD) was used to assess cell viability. Cell sorting was done on a MoFlo flow Cytometer (DakoCytomation, Inc.). Excitation light sources were 100 mW from an argon laser tuned to 488 nm.
and 35 mW from a HeNe laser emitting at 633 nm. Fluorescence signals were collected using amplifiers that reported on a logarithmic scale; forward and side scatter signals were recorded on a linear scale. Sorting was done using the "purify1" mode. Sheath pressure was 60 psi and the sample differential pressure was 0.4 psi or less. Data acquisition, analysis, and compensation were done using Summit software (DakoCytomation). All data were stored as listmode files.

PCR analysis of LOH. Genomic DNA was isolated from sorted cells using the Qiamp blood minikit (Qiagen) or from whole blood by using the PureGene kit (Gentra Systems). DNA was amplified specifically by using primers flanking microsatellite loci D16S521, Kg8, D16S663, D16S3024, D16S3395, and D16S291 on chromosome 16p13. Antisense primers were labeled with fluorescent dyes (6-FAM or HEX, Invitrogen). Primer sequences were obtained from the Genome Database.3 PCR was done in 12.5-μL mixtures containing 10 mmol/L Tris-HCl (pH 8.3), 50 mmol/L KCl, 2.5 mmol/L MgCl₂, 25 μmol/L of each deoxynucleoside triphosphate, 0.8 μmol/L primer, and 0.5 units of AmpliTaq Gold (Applied Biosystems), using a Bio-Rad Mycycler with initial denaturation at 95°C for 2 min, followed by 28 cycles of 95°C (45 s), 55°C (45 s), and 72°C (1 min). PCR products were analyzed on a 3100 Genetic Analyzer (Applied Biosystems). Quotient LOH (QLOH) was calculated by comparing the ratio of fluorescence intensity of each allele in putative LAM (L) cells to that in whole blood (N) from the same patient: (L1/L2)/(N1/N2), where L1 or N1 is the minor allele. L2 and N2 represent the other allele. QLOH values of 0.6 or less were scored as LOH but could also be considered allelic imbalance.

Osteopontin determination. Osteopontin was quantified by ELISA in plasma from LAM patients and normal volunteers using the Human Osteopontin Immunoassay kit from R&D Systems. Samples were taken from a one-time visit, and the patients were selected randomly.

Results

CD44 is expressed in LAM cells. A typical LAM nodule showing areas of proliferating abnormal smooth muscle cells surrounded by hyperplastic type II pneumocytes is shown in Fig. 1A. Whereas LAM cells within the nodule react moderately with anti-CD44 antibody, hyperplastic type II pneumocytes (arrow) and vascular

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3 http://www.gdb.org

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endothelial cells were strongly immunoreactive (rectangle), consistent with previous results (ref. 30; see also Supplementary Fig. S1). Thus, CD44 seems to be present in LAM cells and other cells as well as in lymphocytes (Supplementary Fig. S2). The splice variant CD44v6 is a determinant of metastasis (14), and this variant was detected in proliferating spindle-shaped LAM. Some of the cells reactive with HMB-45 also reacted with anti-CD44v6 (Supplementary Fig. S2). Thus, the proliferating LAM cells were immunoreactive to anti-CD44v6, and only some epithelioid cells immunoreactive for HMB-45 were positive for anti-CD44v6. To identify the lymphatic structures involved in LAM lesions, we used antibody D2-40 that reacts with lymphatic endothelium but not with vascular endothelium (31). The reactivity was specific as nonspecific IgG did not react with LAM cells and type II pneumocytes (Supplementary Figs. S1 and S2).

Cells cultured from LAM lungs contain CD44 and CD44v6 mRNA and are immunoreactive to antibodies. We used the cell mixtures to evaluate CD44 mRNA in the cultured cell populations with RT-PCR designed to detect exons 1 to 5, which are found in the standard form of CD44 and all its splice variants, and we found CD44 mRNA in cells from 10 different patients (Fig. 1B).

Because CD44 is on the cell surface, we immunostained nonpermeabilized LAM cells with the antibody raised against CD44 (clone F-10-44-2) and determined by fluorescence-activated cell sorting (FACS) that ~90% of the cells were immunoreactive (Fig. 1C). The presence of this protein on the surface was corroborated by confocal microscopy showing that immunoreactive CD44 was localized on the cell surface with some seen intracellularly (Fig. 1D).

Approximately 10% to 20% of the cells expressed CD44v6 by flow cytometry (Fig. 1C). The presence of CD44v6 on the cell surface was confirmed by confocal microscopy (Fig. 1D). It was observed that a large fraction of the immunoreactivity of cultured cells was present not only in the periphery of the cells but also intracellularly (Fig. 1D), suggesting that CD44v6 is present in a similar manner as found on normal dermal fibroblasts (Supplementary Fig. S3). Although we cannot rule out a mislocalization of CD44v6 in LAM cells, as has been seen with polycystin-1 in cells with dysfunctional TSC2 (32), we have observed that most of the immunoreactivity with anti-CD44v6 is present intracellularly in cells grown from explanted lungs of LAM patients.

Characterization of cells from LAM patients. We evaluated the presence of CD44 in cells cultured from explanted lungs of 12 patients with sporadic LAM, as described in Materials and Methods. To identify the presence of LAM cells within the heterogeneous population of LAM lung-derived cells, we detected antibody D2-40 that reacts with lymphatic endothelium but not with vascular endothelium (31). The reactivity was specific as nonspecific IgG did not react with LAM cells and type II pneumocytes (Supplementary Figs. S1 and S2).

The presence of different splice variants CD44v6 was assessed in total RNA from 10 lines of cultured LAM cells showing normal presence of two of each alleles as well as abnormal presence of TSC2 alleles (left), FISH for TSC1 (green, arrow) and TSC2 (red, arrowhead) in LAM cell with one (right) or two TSC2 (left) alleles. Bar, 20 μm.

20% to 40% of cells lacking a TSC2 allele as determined by FISH (Fig. 2C compare right and left). We were unable to detect large numbers of cells with alterations of TSC1 nor did we detect loss of TSC2 heterozygosity in these heterogeneous primary cell lines by PCR techniques (see below). The presence of reactive SMA antibodies and HMB–45, and the presence of cells with TSC2 allelic deletion, are consistent with origination of these cells from LAM lung.

Cells cultured from lungs of LAM patients contain CD44v6mRNA and show LOH for TSC2. The presence of different CD44 splice variants was assessed in total RNA from 10 lines of cultured LAM cells. All cell lines tested contained mRNA for the splice variant CD44v6 (Fig. 1B; Supplementary Table S1), as well as for other variants. Because cells within LAM lung nodules reacted with anti-CD44v6 antibodies (Fig. 1), we were prompted to attempt separation of LAM cells based on that characteristic.

To define the relationship between LAM cells and CD44v6, we collected cells with immunoreactive CD44v6 in the surface by sorting (Fig. 3). Although the percentage of cells differed among patients, ~80% of the cells were CD44–CD44v6–, 5% to 15% CD44+CD44v6+, 5% to 10% CD44+CD44v6+, and 2% to 5%
CD44+CD44v6+ (e.g., Fig. 3B). DNA from sorted cells was extracted and tested for LOH of TSC2 using microsatellite loci D16S521, D16S3395, Kg8, D16S663, and D16S291. We observed allelic imbalance that could represent LOH for TSC2 in all the cells positive for CD44v6 (Table 1). Figure 3C shows two examples of LOH for TSC2 in the separated cell populations. The top panel shows PCR products for the microsatellite repeat in the cell mixtures before separation in which no LOH was detected. Although we were not able to detect allelic imbalance or LOH regularly in cell mixtures, we were able to determine that a specific population separated with these antibodies exhibited abnormalities in the TSC2 locus (see Fig. 3C, panels with the arrow). A summary of results for cell lines from 12 patients is shown in Table 1. The data on which our conclusions are based are summarized in Supplementary Table S2. Thus, cells immunoreactive for CD44v6 have abnormalities at the TSC2 locus as evidenced by LOH for TSC2.

Cells immunoreactive with CD44v6 antibody are permeable to 7AAD. As binding of antibodies to CD44 has been correlated with cell death (17), we investigated the viability of cells after exposure to these antibodies using the cell-permeating dye 7AAD (33). Reaction of cells with either CD44 or CD44v6 antibodies increased the number of cells permeable to 7AAD (Fig. 3D). Among the ~20% of nonviable cells, 80% to 90% were those that reacted with CD44v6, and only ~10% reacted with CD44, suggesting that reaction of LAM cells with anti-CD44v6 made them prone to cell death (Fig. 3D). Because the majority of the cells were dead after sorting, further studies were not possible.

Overexpression of CD44 correlated with loss of Tsc2 in rat cells. To assess the presence of CD44 and CD44v6 in cells lacking Tsc2, we used a Tsc2−/− (EEF−/−) cell line from Eker rats (34) and a cell line derived from it that expresses a wild-type Tsc2 gene, cell line EEF+/+ (Tsc2+/+). By confocal immunofluorescence, we showed that there are 14.4 times (Fig. 4A, inset) more CD44 in Tsc2−/− expressed than in Tsc2+/+. The difference in fluorescence intensity was determined on two-dimensional projections (using maximum-pixel-intensity algorithm) of the three-dimensional fluorescence, generated and presented as merged FITC/DAPI channels. The FITC fluorescence intensities are color-coded using a look-up table with a 0 to 4,095 range to create pseudocolor-mapped images (Fig. 4A). FACS analysis and immunohistochemistry also showed a marked difference on the levels of CD44 expression (Fig. 4A and B). CD44 mRNA was present in Tsc2−/− and Tsc2+/+ cells (Fig. 4C) but expression of CD44v6 mRNA was not detected in either cell line (Fig. 4C). CD44 was less abundant in the EEF+/+ than in the EEF−/− cells, suggesting that lack of TSC2 might result in an increased amount of CD44.
Correlation of modulators of CD44v6 splicing and CD44v6 function. To gain insight into the regulation of CD44v6, we considered known modulators of CD44 function and its splicing (i.e., osteopontin). The concentration of osteopontin was determined in plasma of healthy volunteers \((n = 35\); mean age 52.9 ± 2.4 years) and LAM patients \((n = 40\); mean age 50.4 ± 1.7 years). Osteopontin concentration was significantly \((P < 0.001)\) higher in plasma from LAM patients than in that from normal volunteers (Fig. 5). Among the 40 patients studied, eight patients had LAM and TSC. We did not observe a significant difference in the concentration of osteopontin in patients with LAM/TSC (89.8 ± 12.7 ng/mL) and sporadic LAM (72.3 ± 8.6 ng/mL). We determined the relationship of osteopontin with lung function. Osteopontin was not correlated with initial FEV1 \((P = 0.725)\), initial DLCO \((P = 0.250)\), rate of FEV1 decline \((P = 0.113)\), and rate of decline of DLCO \((P = 0.095)\). Thus, levels of osteopontin did not correlate with lung function among LAM patients. Perhaps, there was a trend toward association with rates of decline in lung function with increased osteopontin concentration. The difference between LAM patients and healthy volunteers was age and TSC independent.

We also determined the levels of CD44v6 in plasma of healthy volunteers \((184.5 ± 8.9\, \text{ng/mL}; n = 35)\) and LAM patients \((169.4 ± 11.9\, \text{ng/mL}; n = 40)\). We were not able to determine a correlation between the concentration of CD44v6 and loss of lung function (i.e., initial FEV1, initial DLCO, rate of FEV1 decline, and rate of DLCO decline).

Discussion

Cells with metastatic potential acquire distinct molecular characteristics, which determine the site(s) of metastasis. In addition, at the site of metastasis, the cells often modify their gene expression patterns, resulting in phenotypic changes that can facilitate adaptation to the new environment. To gain insight into the molecular characteristics of the LAM cells that may contribute to metastasis, we investigated whether LAM cells \textit{in vitro} or \textit{in vivo} synthesize CD44v6. Alternative splicing of the cell surface receptor CD44 and its overexpression have been associated with tumor metastasis and progression (12, 13, 27). Among the most studied variants associated with tumor metastasis are CD44v6 and CD44v3 (27). CD44v6 has been associated with many cancers including squamous cell carcinoma (e.g., head and neck, esophagus, lung and skin and cervix and vulva) and adenocarcinoma [e.g., breast, Barrett’s esophagus, lung, gastric, pancreas, colon/rectum, endometrium (uterine), and prostate; ref. 27]. Thus, we decided to evaluate the presence of CD44 and splice variants associated with metastasis in cells grown from patients with LAM and LAM/TSC.

LAM cells in LAM nodules exhibited only a moderate immunoreactivity for CD44, but strong immunoreactivity of other cells (e.g., vascular smooth muscle cells, bronchial epithelial cells, and hyperplastic type II pneumocytes) was noted. The limited reactivity for CD44 standard form could reflect masking of the epitope, as has been suggested for neoplastic cells. It could also represent cleavage of the CD44 standard form by metalloproteases (35) and/or other proteases acting at different sites. The stem region of CD44 contains sites for cleavage by the membrane-associated matrix metalloproteinasases MMP1, MMP9, ADAM10, and ADAM17 (35). MT1-MMP and MMP-2 were found in LAM cells within the lung nodules (36), and other MMPs were overexpressed in patients with LAM. In has also been shown that regulators of MMPs are active and present in LAM lesions (37–39). We also found that the CD44 standard form was present in 90% of the cells grown from explanted lungs of LAM patients. Cells that did not react with the antibody could be those in which the receptor had been cleaved as well as truly negative (non-expressing) cells.

We found a large percentage of LAM cells within the lung nodule that reacted with the CD44v6 antibody, which strengthened our hypothesis that CD44v6 could play a role in LAM and confer metastatic potential to the LAM cells. This observation was corroborated by separation using flow cytometry of a small population of cells immunoreactive with this antibody. Cells that react with anti-CD44v6 antibodies showed LOH for TSC2. We also detected LOH for TSC2 in cells in which CD44v6 was not found (CD44+/CD44v6−; Table 1), but this result was not consistent, which could be due to expression of other splice variants that may also confer metastatic potential (e.g., CD44v3).

Table 1. Determination of LOH for TSC2 in population of cells cultured from explanted lungs of patients with sporadic LAM

<table>
<thead>
<tr>
<th>Cell</th>
<th>(P^*)</th>
<th>CD44+CD44v6+</th>
<th>CD44−CD44v6−</th>
<th>CD44−CD44v6+</th>
<th>CD44−CD44v6−</th>
</tr>
</thead>
<tbody>
<tr>
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<td>6</td>
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<td>R</td>
<td>LOH</td>
<td>LOH</td>
</tr>
<tr>
<td>LAM 2</td>
<td>6</td>
<td>LOH</td>
<td>R</td>
<td>LOH</td>
<td>LOH</td>
</tr>
<tr>
<td>LAM 3</td>
<td>6</td>
<td>R</td>
<td>R</td>
<td>R</td>
<td>LOH</td>
</tr>
<tr>
<td>LAM 4</td>
<td>5</td>
<td>LOH</td>
<td>R</td>
<td>R</td>
<td>R</td>
</tr>
<tr>
<td>LAM 5</td>
<td>1</td>
<td>LOH</td>
<td>R</td>
<td>R</td>
<td>LOH</td>
</tr>
<tr>
<td>LAM 6</td>
<td>4</td>
<td>R</td>
<td>R</td>
<td>R</td>
<td>LOH</td>
</tr>
<tr>
<td>LAM 7</td>
<td>3</td>
<td>LOH</td>
<td>R</td>
<td>R</td>
<td>LOH</td>
</tr>
<tr>
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<td>4</td>
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<td>R</td>
<td>LOH</td>
<td>LOH</td>
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<tr>
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<td>LOH</td>
<td>R</td>
<td>LOH</td>
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<td>1</td>
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<td>R</td>
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Abbreviation: R; retention of heterozygosity.

*Number of times that the cells have been replated before cell sorting.
CD44 is a protein that exists, as a result of alternative mRNA splicing, in at least 10 different variants with different functions (12). The observation of immunoreactivity for CD44v6 in cell nuclei (data not shown) might reflect its cleavage and subsequent internalization. This was shown for CD44 by presenilin/γ-secretase at the intramembrane domain, which generated a cytoplasmic fragment that served as a transcriptional regulator (40, 41). CD44v6 seems to represent a cell surface marker expressed by LAM cells that showed LOH for TSC2 and could help in their identification. It may also play a pivotal role in pathogenesis of the disease.

The amounts of CD44 and its splice variants at the cell surface depend, in part, on growth factor regulation of CD44 RNA splicing. Although levels of CD44 seemed to correlate with loss of TSC2 in Fig. 4, the presence of CD44v6 did not correlate with loss of TSC2 in

![Figure 4](image-url)

**Figure 4.** CD44s in Eker rat cells. A, CD44 and CD44v6 immunoreactivity of Tsc2–/– and Tsc2+/+ cells. The stronger reactivity is seen in Tsc2–/– cells than in Tsc2+/+, which is quantified in the inset, showing that the difference is 14-fold. This experiment was repeated twice. B, cytometric analyses of Eker rat cells (red) Tsc2+/+ and Tsc2−/− with isotype (negative) control (blue). C, the presence of CD44 mRNA in cells derived from tumors of Eker rat Tsc2+/+ and Tsc2−/−. Detection of mRNA for CD44v6 (bottom) in cells derived from LAM patients (positive control) and Eker rat tumor Tsc2+/+ and Tsc2−/−. NC, negative control (minus reverse transcriptase).

![Figure 5](image-url)

**Figure 5.** Concentration of osteopontin in plasma of LAM patients and normal volunteers. A, concentration of osteopontin (OPN) in plasma of 40 LAM patients who had not undergone lung transplantation compared with that of 35 healthy female volunteers. There was a significant difference in the levels of osteopontin, with a P value of 3.6 × 10⁻⁵. B, correlation between the levels of osteopontin (ng/mL) with the initial FEV1 and initial DLCO. C, correlation between the levels of osteopontin (ng/mL) with the rate of FEV1 decline and rate of DLCO decline.
cells from the Eker rat. Thus, it seems that the LAM cells from human lung are different. CD44 splicing has been correlated with different signaling mechanisms, including those mediated by osteopontin and epidermal growth factor (13, 20, 42). In fact, the mean plasma concentration of osteopontin was higher in LAM patients than in normal volunteers (Fig. 5). Because osteopontin binds to CD44, elevated levels of osteopontin could influence cell migration (43), consistent with a potential role in disease progression. In fact, the mean plasma concentration of osteopontin was higher in LAM patients than in normal volunteers (Fig. 5).

CD44 has a single transmembrane span with a COOH-terminal cytoplasmic tail that binds the cytoskeletal protein ankyrin and members of the band 4.1 superfamily, including the tumor suppressor NF2 that encodes merlin (12) and other proteins such as ezrin. It also associates with multiple receptors (44, 45). It may be relevant that ezrin, which has been associated with tumor progression, can interact with hamartin. Thus, a dysfunctional TSC2 as exists in LAM cells could affect the function of TSC1 and possibly ezrin (46).

It was recently shown that the extracellular portion of CD44, including the v6 portion, sequesters Fas ligand, resulting in inhibition of apoptosis (17). Binding of Fas ligand to the CD44v6 domain was competed by anti-CD44v6. It has been shown that antibodies to CD44 can trigger apoptosis, but the mechanisms have not been clearly identified; based on the role of CD44, however, it is possible that binding of antibody to the extracellular portion of CD44 triggers apoptosis by direct signaling events and by altering cell-to-cell and cell-to-extracellular matrix interactions.

The application of antibodies against CD44v6 in cancer diagnosis and treatment has been of interest. Binding of antibodies to CD44 has been associated with both apoptosis and cell proliferation. We observed that >80% of cells that reacted with CD44v6 antibodies were permeable to the dye 7AAD, which is presumed to indicate lack of viability. The discovery of CD44v6 in LAM cells and the potential to trigger cell death with this antibody prompt us to suggest that CD44v6 might be a therapeutic target in LAM.

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