Mesothelin Overexpression Promotes Mesothelioma Cell Invasion and MMP-9 Secretion in an Orthotopic Mouse Model and in Epithelioid Pleural Mesothelioma Patients

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Abstract

Purpose: Mesothelin (MSLN) is a tumor-associated antigen, being investigated as a biomarker and therapeutic target in malignant pleural mesothelioma (MPM). The biologic function of MSLN overexpression in MPM is unknown. We hypothesized that MSLN may promote tumor invasion in MPM, a tumor characterized primarily by regional aggressiveness and rare distant metastases.

Experimental Design: Human and murine MPM cells with MSLN forced expression and short hairpin RNA knockdown were examined for proliferation, invasion, and matrix metalloproteinase (MMP) secretion. The influence of MSLN overexpression on MPM cell invasion was assessed in an orthotopic mouse model and in patient samples.

Results: MSLN expression promotes MPM cell invasion and MMP secretion in both human and murine MPM cells. In an orthotopic MPM mouse model characterized by our laboratory, MPM cells with MSLN overexpression preferentially localized to the tumor invading edge, colocalized with MMP-9 expression, and promoted decreased survival without an increase in tumor burden progression. In a tissue microarray from epithelioid MPM patients (n = 139, 729 cores), MSLN overexpression correlated with higher MMP-9 expression at individual core level. Among stage III MPM patients (n = 72), high MSLN expression was observed in 26% of T2 tumors and 51% of T3 tumors.

Conclusions: Our data provide evidence elucidating a biologic role for MSLN as a factor promoting tumor invasion and MMP-9 expression in MSLN expressing MPM. As regional invasion is the characteristic feature in MSLN expressing solid cancers (MPM, pancreas, and ovarian), our observations add rationale to studies investigating MSLN as a therapeutic target.

Introduction

Mesothelin (MSLN) is a 40-kDa glycoprotein overexpressed in mesothelioma, pancreatic, and ovarian cancers—malignancies characterized by regionally aggressive phenotypes and poor prognosis (1–3). Although MSLN is being investigated as a tumor biomarker and therapeutic target in malignant pleural mesothelioma (MPM), the biologic role of MSLN remains unexplored (4–9).

MSLN is highly expressed in epithelioid MPM, the most common MPM subtype constituting 80% of patients. Only 3% of epithelioid MPM patients showed distant metastases in multiple series published by our group (10–12). Despite an absence of distant metastases, a majority of epithelioid MPM patients are not eligible for surgical resection due to advanced T-stage (T3 tumors are unresectable due to local invasion into the endothoracic fascia, mediastinal fat, chest wall, or pericardium compared with T2 tumors without invasion; refs. 13–15). Matrix metalloproteinases (MMP), a family of endopeptidases capable of degrading extracellular matrix, have been shown to be elevated in MPM and are known to increase invasive potential in MPM cells (16). Furthermore, asbestos exposure, a causative agent in MPM, is known to upregulate both MSLN and MMP-9 secretion in experimental models of asbestosis (17).

In this study, we explored the role of MSLN in tumor invasion and its relationship to MMP-9 secretion using human and murine mesothelioma cells both in vitro and in vivo as well as in clinical specimens from epithelioid MPM patients, known to overexpress MSLN. We show for the first time that MSLN promotes MMP-9 expression as well as tumor invasion shown by MSLN forced overexpression and...
**Translational Relevance**

Mesothelin (MSLN) is a cancer-associated antigen overexpressed in malignant pleural mesothelioma (MPM) and other regionally invasive malignancies. We hypothesized that MSLN expression promotes MPM cell invasion. In human and murine MPM cells with MSLN overexpression and knockdown, we show that MSLN promotes MPM cell invasion. In an orthotopic MPM mouse model characterized by our laboratory, we show that MSLN overexpressing MPM cells preferentially localize to the tumor invading edge and decrease survival without an increase in tumor burden progression. Both *in vitro* and *in vivo* and in MPM patient samples, we show an association between MSLN overexpression, tumor invasion, and matrix metalloproteinases, a family of enzymes that facilitate tumor cell invasion and known to be upregulated in MPM. As regional invasion is a characteristic feature in MSLN expressing solid cancers (MPM, pancreas, and ovarian), these findings add support for studies investigating MSLN as a therapeutic target.

confirmed by short hairpin RNA (shRNA) knockdown experiments in mesothelioma cells. To further elucidate MSLN biology in an appropriate tumor microenvironment, we developed and characterized an orthotopic MPM mouse model. With this model, we show that MSLN expressing MPM cells are invasive, express MMP-9 on the invasive tumor edge, and decrease overall survival independent of tumor cell proliferation or metastasis. Furthermore, our clinical observations from a large cohort of epithelioid MPM patients show that MSLN expression correlates with MMP-9 expression. The results reported herein provide evidence that MSLN also plays an important role in MPM biology and suggest the MMP pathway as a mediator of invasiveness in MSLN expressing MPM.

**Materials and Methods**

**Cell lines and culture**

MSTO-211H (human pleural mesothelioma) and AB12 (murine mesothelioma line) were obtained from American Type Culture Collection and CellBank Australia, respectively. MSTO-211H cells were maintained in RPMI-1640 media supplemented with 10% FBS, 100 units/mL penicillin, and 0.75 mL RPMI-1640 with 10% FBS in the bottom chamber. MSTO-211H cells with or without MSLN expression were reinfected 24 hours later. The human MSLN-variant 1 was isolated from a human ovarian cancer cell line (OVCAR-3). Reverse transcriptase PCR (RT-PCR) synthesis of full-length cDNA of human MSLN was done using SuperScript III One-Step RT-PCR System with Platinum Taq High Fidelity Kit. Plasmid DNA was isolated, subcloned into a SFG retroviral vector, confirmed by sequencing, and used to stably transduce MSLN. For experiments comparing MSLN-transduced cells to MSLN-negative cells, transduction control was done with a GFP-Firefly Luciferase vector. For all experiments, a stably transduced population of cells was used with confirmation of unchanged MSLN expression by flow cytometry and Western blot analysis.

**Mesothelin knockdown with MSLN-specific shRNA**

To obtain a stable cell line with decreased murine MSLN expression, 3 predesigned siRNA oligonucleotides and complementary murine MSLN shRNA sequences were obtained (Ambion), ligated into the pSilencer 2.1-U6 hygro plasmid (Ambion), and transfected into the AB12 cell line with calcium phosphate. After 2 weeks of selection with 500 μg/mL hygromycin (Invitrogen) the AB12 cell line showing greatest murine MSLN silencing by flow cytometry, qPCR analysis, and Western blot was selected for subsequent experiments and is denoted by AB12shRNA. AB12 cells were also transfected with scramble shRNA as a control.

**Flow cytometry**

Fluorescence-activated cell sorting (FACS) was carried out following retroviral transductions using a FACSAria (BD Biosciences) cytometer to sort for a pool of highly transduced cells. Human MSLN expression was detected using a PE-conjugated or APC-conjugated anti-human MSLN rat IgG2a (R&D systems). Murine MSLN expression was detected with an anti-mouse MSLN rat IgG2a primary antibody (MBL). Subsequent flow cytometry for GFP and MSLN expression analysis was done on either FACSCaliber or LSRII cytometers (BD Biosciences) and analyzed using FlowJo (TreeStar) analysis software.

**Cell proliferation assays**

MSTO-211H cells with or without MSLN expression were plated in 6-well tissue culture plates at a density of 1 x 10⁵ cells/3 mL/well and were counted on days 1, 2, 4, and 6. At each time point, cells were counted in triplicate using trypan exclusion after brief trypsinization. Cell number versus time was plotted for each cell line and compared at time points using a Student *t* test.

**Invasion and migration assays**

BD BioCoat Matrigel Invasion Chamber (BD Biosciences) assays were done in 24-well plates using polyethylene terephthalate inserts (8-μm pores) without Matrigel (for migration) and with Matrigel coating (for invasion assays). Cells were seeded in the upper well of the chamber (5 x 10⁴ to 1 x 10⁵) in 0.5 mL RPMI-1640 without FBS and 0.75 mL RPMI-1640 with 10% FBS in the bottom chamber. Migration was assessed at 12 hours and invasion at 24 hours.

**Establishment of stably transduced cell lines**

GFP-Firefly Luciferase fusion was cloned into a SFG retroviral vector and transfected into H29 cells with calcium phosphate. MSTO-211H was plated in 24-well plates 24 hours before transduction. Filtered virus was added to cells permeabilized with 8 μg/mL polybrene (Sigma-Aldrich) and reinfected 24 hours later. The human MSLN-variant 1 was isolated from a human ovarian cancer cell line (OVCAR-3). Reverse transcriptase PCR (RT-PCR) synthesis of full-length cDNA of human MSLN was done using SuperScript III One-Step RT-PCR System with Platinum Taq High Fidelity Kit. Plasmid DNA was isolated, subcloned into a SFG retroviral vector, confirmed by sequencing, and used to stably transduce MSLN. For experiments comparing MSLN-transduced cells to MSLN-negative cells, transduction control was done with a GFP-Firefly Luciferase vector. For all experiments, a stably transduced population of cells was used with confirmation of unchanged MSLN expression by flow cytometry and Western blot analysis.
for MSTO-211H. For AB12 cells, migration was assessed at 6 hours and invasion at 20 hours. Nonmigrating or non-invading cells were removed from the upper membrane surface of the insert using a cotton swab. The insert membrane was fixed and stained using the Diff-Quik staining system and mounted on glass slides. Stained cells were counted in 10 high-power fields in predetermined areas of the membrane.

**Orthotopic pleural mesothelioma animal model**

To develop the orthotopic mouse model of pleural mesothelioma, female severe combined immunodeficient/beige or BALB/c mice (Taconic) at 6 to 10 weeks of age were used. All procedures were done under approved Institutional Animal Care and Use Committee protocols. Mice were anesthetized using inhaled isoflurane and oxygen. Direct intrapleural injection of $1 \times 10^6$ tumor cells in 200 μL serum-free media via a right thoracic incision was administered to establish orthotopic MPM tumors as previously described (18–21). For experiments to establish heterogeneously MSLN expressing tumors, mice were inoculated by direct pleural injection with a mixture of $1 \times 10^5$ MSTO-211H expressing and deficient in MSLN.

**Histology and immunostaining**

Histopathologic evaluation of tumors was carried out following hematoxylin and eosin (H&E) staining of paraffin-embedded, 4% paraformaldehyde fixed tissue samples. For angiogenesis, CD34 rat monoclonal antibody (5 μg/mL; eBioscience) was incubated for 7 hours, followed by 16 minutes with (1:200) biotinylated rabbit anti-rat IgG (catalog #BA-4000; Vector Labs). Rat IgG2a (5 μg/mL) was used as an appropriate isotype negative control. For lymphangiogenesis, goat polyclonal LYVE-1 antibody (1 μg/mL; R&D Systems) was incubated for 3 hours, followed by 60 minutes with biotinylated rabbit anti-goat IgG (ABC kit from Vector Labs). The protocols for immunofluorescence detection using Tyramide–Alexa Fluor 488 (Invitrogen) or Tyramide–Alexa Fluor 568 (Invitrogen) for CD34 and LYVE-1, respectively, were established and done at the MSKCC Molecular Cytology Core Facility using a Discovery XT automatic processor (Ventana Medical Systems). Immunohistochemistry (IHC) for human MSLN was done with a mouse anti-human MSLN IgG (1:100; Vector Labs) using the Ventana platform. IHC for MMP-9 was done by Premier Laboratories using polyclonal rabbit anti-human MMP-9 (Dako). IHC for MMP-7 was done using a mouse IgG2b anti-human MMP-7 clone ID2 (Abcam) in a 1:100 dilution. Placenta with cytoplasmic staining in the villi was used as the MMP-7 positive control.

**Quantitative bioluminescence imaging**

*In vitro* standardization was done using GFP-Firefly Luciferase expressing MSTO-211H cells with and without MSLN expression. Serially diluted cells were plated in 96-well tissue culture plates ($1.6 \times 10^4$ to $2.5 \times 10^5$ cells/100 μL/well). Twenty minutes after the addition of 100 μL of luciferin (15 mg/mL; Caliper Life Sciences), plates were imaged using the Xenogen IVIS 100 Imaging System (Caliper Life Sciences). Cell number versus total bioluminescence imaging (BLI) flux (photons/s) was evaluated by Pearson’s correlation.

*In vivo* BLI in tumor-bearing mice was done using a single intraperitoneal dose of 150 mg/kg α-luciferin. Mice were imaged with the Xenogen IVIS 100 Imaging System 20 minutes following α-Luciferin injection. Images were acquired for 5 to 30 seconds depending on signal strength. BLI data were analyzed using Living Image 2.60 software and BLI signal reported as total flux (photons/s).

**Gene expression analysis**

Gene expression profiles were compared between MSLN expressing and nonexpressing MSTO-211H cells. Cell pellets were collected in triplicate and snap frozen from 3 separate plates for each cell line. The Genomics Core Laboratory at MSKCC collected, processed, and hybridized RNA samples to Illumina Humanref-6 BeadChips. Using Bioconductor package LIMMA, sample probesets were normalized to control probesets to minimize inter-beadchip variability. Individual gene expression was screened for significance using a false discovery rate threshold of less than 0.05 and a fold change threshold of 1.5. Gene set enrichment analysis (GSEA; ref. 22) was carried out to determine biologic pathways related to MSLN overexpression.

**Matrix metalloproteinase assays**

MMP secretion by tumor cells *in vitro* was quantified by multiplex bead assays (Millipore) for MMP-1, MMP-2, MMP-7, and MMP-9 on a Lumexin 100 xMAP with internal quality control standards for each analyte. A total of $1 \times 10^5$ cells in 1 mL media were plated in 24-well tissue culture plates and allowed to grow for 24 hours, media was then removed, washed with PBS, and media replaced with 0.5 mL RPMI-1640 without FBS. Cell supernatant MMP-9 secretion was quantified by multiplex bead assay for MMP-9 (Millipore). Supernatant was collected 12, 24, and 36 hours after media exchange and stored at $-80^\circ$C until analyzed.

**Epithelioid MPM tissue microarray and IHC**

Patients diagnosed with epithelioid MPM between 1989 and 2009 at Memorial Sloan-Kettering Cancer Center were...
included. For each of the 139 patients with available specimens, all H&E slides (median 9, range 1–43) were reviewed. Representative blocks were selected to construct a tissue microarray (TMA) by taking 9 representative cores (0.6 mm) from each patient tumor block and ensuring at least 6 complete tumor cores. Five micrometer–sections were cut from the TMA and stained by specific antibodies (MSLN: Vector, 1:200 dilution; MMP-9: Oncogene Science, 1:200 dilution). Grading of MSLN and MMP-9 intensity was carried out on separate occasions by a pathologist who was blinded to the clinical data as follows: 0 (absent stain), 1 (weak expression), 2 (moderate expression), and 3 (strong expression). The distribution of MSLN-positive tumor cells from all tumor cells found in a single core was graded as 0 (absent), 1 (1%–50%), and 2 (51%–100%). The sum of the MSLN stain intensity and distribution grades was used to determine a total MSLN score ranging from 0 to 5 at the core level. MSLN score for each patient was then determined using the average of all tumor cores.

We examined the correlation between MMP-9 and MSLN expression using Mantel–Haenszel correlation statistics. The P values were adjusted through bootstrap to account for the fact that each of the 139 patients can contribute with more than one core to the analysis, leading to a total of 721 cores. The correlation between increasing MSLN expression and T stage was determined using the Fisher exact test.

Results

Mesothelin expression promotes invasion and migration in vitro independent of tumor cell proliferation

We initially investigated the effect of MSLN overexpression on MPM by comparing MSTO-211H cells without MSLN expression to those stably transduced to overexpress MSLN (Fig. 1A) and AB12 cells with natural MSLN expression or knockdown (Fig. 1B). We noted no morphologic differences in cultured cells overexpressing MSLN, and cell-counting assays showed no effect of MSLN expression on MSTO-211H proliferation (Fig. 1C). Similarly, we observed no differences in morphology or log-phase growth rates between AB12 cells transfected with control scramble shRNA or MSLN-specific shRNA (data not shown). To examine whether MSLN expression promoted changes in
MSTO-211H proliferation during periods of cellular stress, experiments were repeated with serum-starved (2% fetal calf serum) media; no differences were observed (data not shown). Because local invasion is the characteristic clinical feature of mesothelioma, we evaluated in vitro cell migration and invasion using a standard Boyden chamber assay. MSLN overexpression in MSTO-211H significantly increased cell migration (2.56-fold, \( P < 0.001 \)) and invasion (1.54-fold, \( P < 0.001 \)) compared with non-MSLN expressing MPM cells (Fig. 1D and E left panel). To confirm our observations of MSLN influence on invasion, we next examined the effect of MSLN-knockdown in the AB12 cell line that natively expresses MSLN (Fig. 1B). Decreased MSLN expression resulted in decreased migration (2.14-fold, \( P < 0.001 \)) and invasion (2.35-fold, \( P < 0.0001 \); Fig. 1E right panel). These results showed that MSLN overexpression promotes invasion in MPM cells.

**Development and characterization of a clinically relevant orthotopic mouse model of MPM to investigate mesothelin biology**

Having noticed that MSLN overexpression promotes MPM invasion and migration in vitro, we next sought to investigate the biology of MSLN within an appropriate tumor microenvironment. To evaluate local pleural and chest wall invasion, an important clinical factor in the progression of MPM, which cannot be fully simulated in subcutaneous or intraperitoneal mouse models, we developed an orthotopic mouse model by directly inoculating MPM cells into the pleural space (18, 20, 23, 24). Resultant tumors mimicked human MPM as shown by (i) gross pleural and mediastinal tumor distribution seen on necropsy and MRI (Fig. 2A); (ii) histology showing tumors growing along visceral and parietal pleural surfaces, frequent chest wall and diaphragmatic invasion, and rare lung invasion (Fig. 2B); (iii) frequent metastases to mediastinal lymph nodes confirmed by histology (Fig. 2C) without distant metastases; (iv) retention of characteristic MPM tumor markers including WT-1, Calretinin, and MSLN even at late stages of disease although TTF-1, a marker of lung adenocarcinoma, remained negative (Fig. 2D); and (v) extensive neolymphangiogenesis throughout the MPM tumor specimens by CD34 and LYVE-1 immunofluorescence for angio- and lymphangiogenesis, respectively (Fig. 2E; MPM is known to be a well vascularized tumor with high levels of VEGF secretion; refs. 25, 26).
These findings not only confirmed that our orthotopic pleural mesothelioma mouse model resembles human MPM, but also provided an appropriate in vivo tumor microenvironment to investigate MPM tumor cell invasion.

Quantitative bioimaging monitors the effect of mesothelin on tumor progression in vivo

Using our orthotopic MPM mouse model, we first sought to evaluate whether MSLN imparts a growth advantage in vivo. Unlike subcutaneous flank tumors, which are easily accessible for serial monitoring, evaluating the effect of MSLN expression on pleural tumors required an accurate noninvasive method to serially evaluate tumor burden and progression. To do so, we validated bioluminescent imaging (BLI) as a quantitative modality to assess tumors in the pleural model. First we carried out an in vitro BLI standardization with MSLN expressing and nonexpressing MPM cells stably transduced express a GFP-Firefly Luciferase fusion gene (Fig. 3A). Our results showed that BLI signal correlated with number of cells for both MSLN-negative (Pearson $r = 0.99$, $P < 0.0001$) and positive (Pearson $r = 0.99$, $P < 0.0001$) cell lines (Fig. 3B). We next validated BLI as an accurate method to monitor tumor progression within the orthotopic MPM mouse model by serially comparing BLI signal to MRI tumor volume averaging, the gold standard for tumor volume assessment (27). BLI signal from the engrafted pleural tumors correlated with MRI tumor volume over a wide range of tumor burdens ($r = 0.86$, $P < 0.0001$; Fig. 3C and D). Thus, this orthotopic MPM mouse model allows for quantifying tumor burden and monitoring tumor progression in the pleural tumor microenvironment (21).

Mesothelin expression promotes tumor invasion in vivo and decreases survival without affecting tumor proliferation

Mice inoculated with equivalent intrapleural MSLN expressing and nonexpressing MPM cells were compared for in vivo tumor progression. Serial BLI was used to monitor tumors in mice and revealed no differences in tumor burden between tumors with or without MSLN expression at any point in disease progression (Fig. 4A). Despite equivalent tumor progression rates and tumor burden, mice with MSLN expressing MPM experienced significantly decreased survival compared with mice with MSLN-negative tumors (29 vs. 37 days, $P = 0.001$; Fig. 4B). These results of decreased survival with MSLN expression were reproduced in our syngeneic orthotopic MPM model using BALB/c mice with murine AB12 MPM cells. Mice inoculated with AB12 wild-type (MSLN expressing) tumors had a median survival of 30 days compared with AB12 shRNA (MSLN knockdown), which survived the 60-day study period ($P < 0.001$). We further evaluated AB12 survival by transducing MSLN expressing AB12 cells with mouse MSLN vector to express MSLN at even higher levels (AB12 M). We observed decreased survival of mice inoculated with these high MSLN expressing AB12 cells compared with AB12 wild-type cells (24 vs. 30 days, $P = 0.005$; Supplementary Fig. S1).

Systematic histologic evaluation of necropsy specimens showed MSLN expressing tumors to have qualitatively increased local tumor invasion of chest wall, diaphragm, and mediastinal structures as compared with tumors without MSLN. To further investigate the association between MSLN expression and local tumor invasion in vivo, we evaluated mice with orthotopic MPM tumors heterogeneously expressing MSLN. Heterogeneously expressing

![Figure 3. Validation of BLI in the mouse model to quantitatively monitor MSLN effect on tumor burden and progression. A, flow cytometry dot plots showing human MPM cells (MSTO-211H) stably transduced to express GFP-Firefly Luciferase gene (x-axis) in both MSLN-negative (left plot) and positive (right plot) cells. B, in vitro validation of BLI shows that BLI signal correlates with cell number over a wide range for both MSLN-negative ($r = 0.99$, $P < 0.0001$) and MSLN-positive ($r = 0.99$, $P < 0.0001$) cells. C, in vivo BLI validation by correlating BLI signal (left column) to tumor volume by MRI volume averaging (right column) in mice with orthotopic MPM tumors. D, BLI signal correlated strongly with tumor volume over a wide range of tumor progression in mice with pleural mesothelioma with and without MSLN expression.](http://www.aacrjournals.org/clinica/18/05/2483)
tumors were established by inoculating mice with a mixture of MSLN expressing and MSLN-deficient MSTO-211H cells. These tumors uniformly showed clustering of MSLN expression at the advancing (invading) edge as determined by MSLN IHC (Fig. 4C and D top panel).

Mesothelin expression promotes MMP secretion, which colocalizes at the invading tumor edge

Having observed MSLN expression at the invasive edge of MPM tumors, we next investigated whether pathways known to cause tumor invasion were upregulated in MPM cells overexpressing MSLN. GSEA (22) of gene array data comparing human MPM cells (MSTO-211H) with or without MSLN expression showed that MSLN expression increased the expression of genes known to regulate MMP secretion ("SA-MMP-cytokine connection"; hypergeometric $P < 0.001$; Supplementary Table S1). To investigate these findings, we evaluated MMP secretion in cultured human MSTO-211H and murine AB-12 MPM cells. Using multiplex bead assays for human and murine MMPs, we found that MSLN-overexpressing MSTO-211H cells produced significantly increased MMP-1, 2, 7, and 9 compared with MSLN-deficient MPM cells ($P < 0.01$, Fig. 5A). To confirm that MSLN expression has a direct relationship to MMP secretion, we examined the effect of MSLN knockdown by shRNA using the MSLN expressing murine MPM cell line AB12; decreased MSLN expression was confirmed by flow cytometry (Fig. 1B) and qPCR (relative-fold MSLN expression 0.483 $\pm$ 0.29). We found that cell supernatant secretion of MMP-9 was significantly decreased following MSLN knockdown in the AB12 cell line ($P < 0.0001$, Fig. 5B).

We then evaluated expression of MMP-9 in MPM tumors in vivo. We carried out IHC for MMP-9 on heterogeneously MSLN expressing MPM cells showing increased MSLN expression at the advancing tumor edge in tumor nodules on both diaphragm (top image asterisk) and chest wall (bottom image arrowhead). MMP-9 IHC shows increased MMP-9 expression at the leading invasive edge (bottom image arrow) compared with baseline MMP-9 expression in the areas of tumor with low levels of MSLN expression (bottom image asterisk).

Mesothelin is overexpressed in 90% of epithelioid MPM patients

As MSLN IHCs are not routinely done in MPM patients, the only MSLN expression data available to date are from tumors were established by inoculating mice with a mixture of MSLN expressing and MSLN-deficient MSTO-211H cells. These tumors uniformly showed clustering of MSLN expression at the advancing (invading) edge as determined by MSLN IHC (Fig. 4C and D top panel).
small case series (33–49 patients) comprising varying histologic subtypes (28, 29). Therefore, we first evaluated MSLN expression in a large uniform cohort of epithelioid MPM patients (n = 139). MSLN is overexpressed in 90% of epithelioid MPM patients (score 1–5), with strong expression present in 29% of epithelioid MPM patients (score 4–5; Fig. 6A).

Mesothelin overexpression is associated with high MMP-9 expression in human epithelioid MPM specimens

To determine the relationship between MSLN and MMP-9 expression in human tumor samples, a TMA (6 tumor cores per patient) derived from 139 surgically resected epithelioid MPM tumors (Supplementary Table S2) was examined by IHC (Fig. 6A). We detected a strong correlation between MMP-9 expression and MSLN intensity at individual core level—observing increased levels of MMP-9 expression with increasing MSLN expression (Fig. 6B, P < 0.001). These clinical findings supported our preclinical observations associating MSLN expression with increased MMP levels.

Mesothelin intensity and distribution in epithelioid MPM patients

We examined MSLN expression score (sum of intensity and distribution) in stage III epithelioid MPM patients. To avoid confounding effects due to heterogeneous clinical stages and neoadjuvant therapy, we selected a uniform cohort of 72 stage III patients with no prior therapy [T1 = 1 (excluded), T2 = 17 and T3 = 54 patients]. In this cohort, a high MSLN score (4–5) is observed in 26% versus 51% for T2 and T3 patients, respectively (Fig. 6C, P = 0.05). These data were notable because increasing T-stage represents an important clinical distinction of tumor invasion.

Discussion

Tumors that are known to express MSLN including MPM, pancreatic, and serous ovarian cancers are characterized by locoregional aggressiveness (12). Local tumor invasion is the primary cause of morbidity in patients with MPM and the factor most often preventing tumor resectability (11, 14). The majority of studies in MPM and other regionally aggressive malignancies have focused on MSLN as a
tumor biomarker (8, 9) and therapeutic target, although none have evaluated the influence of MSLN on tumor biology. Our study provides evidence showing for the first time a correlation between MSLN and MMP expression as well as regional tumor aggressiveness and invasion in MPM cells, in an orthotopic MPM model, and in epithelioid MPM patients.

It is well documented that MMP-mediated degradation of extracellular matrix proteins facilitates cancer cell migration and invasion (30, 31). MMPs have been implicated in the tumor progression of several cancers including breast (32, 33), pancreatic (34), lung (35), ovarian (36), and mesothelioma (37, 38). In a recent publication, Chang and colleagues showed an associated between MMP-7 and invasion in ovarian cancer (39), we found no correlation in human MPM tumor specimens between MMP-7 and either MSLN expression or tumor stage (data not shown). Our data suggest that although secretion of multiple MMPs is increased in association with MSLN in vivo, MMP-9 plays a predominant role in the in vivo human MPM tumor. The results reported herein highlight MMP-9 as a potential pathway for MSLN-mediated MPM invasiveness.

MSLN is a glycophosphoinositol (GPI)-anchored cell-surface protein, for which signal transduction depends upon associating with accessory proteins. GPI-anchored proteins such as T-cadherin can transduce signals that modulate cell migration and invasion by mechanisms mediated through direct interactions with integrin-linked kinase (44) and integrin β3 (45). Data from our gene array studies show an association between integrin β4 and MSLN overexpression in MPM (Supplementary Table S1). We have confirmed the expression of integrin β4 in MSLN overexpressing MPM cells by flow cytometry (Supplementary Fig. S2). Integrin β4 is expressed by epithelioid MPM (46), and published literature implicates integrin β4 expression with tumor cell migration, invasion, and MMP expression (47). Further studies to better define the association between MSLN and integrin β4 may ultimately help elucidate the relationship of this pathway to MPM tumor cell migration, invasion, and MMP expression.

Figure 6. TMA constructed from 139 epithelioid MPM tumors reveals an association between MSLN expression and MMP-9 overexpression. A, representative TMA cores depicting strong or low MSLN and MMP-9 expression (bar = 50 μm). B, MMP-9 expression increases as the proportion of tumor cores showing strong (black) and moderate (gray) expression of MMP-9 increases as compared with weak (hatched) or absent (white) MSLN expression (P < 0.001). C, in patients with invasion into the endothoracic fascia, mediastinal fat, chest wall, or pericardium (T3-tumors), there is a trend toward increased strong MSLN expression (black; score 4–5) compared with absent–moderate expression (gray; score 0–3) when compared with T-2 tumors (P = 0.05).

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Review of the literature reveals other putative mechanisms for influence of MSLN on tumor phenotype besides the association with MMP expression that we have shown. Functionally, investigators have shown high-affinity binding between MSLN and CA-125 present on mesothelial cells (48)—an interaction thought to contribute to peritoneal migration of ovarian cancer. Uehara and colleagues (49) have found MSLN overexpression to promote anchorage-independent growth and resistance to anoikis in association with suppression of proapoptotic Bim. Chang and colleagues (50) have implicated the phosphoinositide 3-kinase

Figure 6. TMA constructed from 139 epithelioid MPM tumors reveals an association between MSLN expression and MMP-9 overexpression. A, representative TMA cores depicting strong or low MSLN and MMP-9 expression (bar = 50 μm). B, MMP-9 expression increases as the proportion of tumor cores showing strong (black) and moderate (gray) expression of MMP-9 increases as compared with weak (hatched) or absent (white) MSLN expression (P < 0.001). C, in patients with invasion into the endothoracic fascia, mediastinal fat, chest wall, or pericardium (T3-tumors), there is a trend toward increased strong MSLN expression (black; score 4–5) compared with absent–moderate expression (gray; score 0–3) when compared with T-2 tumors (P = 0.05).
pathway in MSLN-associated antiapoptosis. Perturbations in Wnt1/β-catenin or Ras pathways lead to the expression of MSLN in mammary (51) and pancreatic adenocarcinomas (52). Overexpression of Wnt1 in colorectal cancer cells, which is also often constitutively activated in mesothelioma, ovarian, and pancreatic cancers, was associated with MSLN expression (43). However, these mechanisms neither explain nor provide a pathway for the specific observation of MSLN-associated local tumor invasion, a clinically important characteristic of MSLN expressing cancers.

Tumor invasion has typically been studied using the Boyden chamber and scratch assays in vitro. We have established a novel orthotopic MPM mouse model wherein tumor invasion into diaphragm, mediastinal fat, and pericardium mimic human MPM and have shown that MSLN overexpressing cells localized to the invading edge. Furthermore, to show that MSLN is indeed associated with invasion, we have used a uniform cohort of stage III epithelioid MPM patients differing by T-stage and confirmed our preclinical observations of MSLN and MMP-9 expression at tumor core level. In the experience of our group and others, MPM local invasion and regional aggressiveness is a major factor preventing surgical resection with curative intent (14, 53): 40% of patients are deemed unresectable on the operating table in spite of extensive preoperative imaging studies. On the basis of our observations, we are currently investigating the role of MSLN level in both tumor tissue and patient serum to predict resectability for patients with a low clinical “T” stage based. In addition, recent cancer vaccine clinical trials in patients with pancreatic cancer have shown the beneficial effects of MSLN-specific immune responses in prolonging survival (54, 55)—a feature currently being explored for the development of MSLN-targeted immunotherapies by our group and others (56–58).

This study provides further rationale for investigating MSLN-tar-

tified immunotherapies by our group and others (56–58).

This study provides further rationale for investigating MSLN-targeted therapies in MPM, as MSLN provides a molecular target that is commonly expressed in human epithelioid MPM and promotes tumor invasion. In summary, this study is the first to show an association between MSLN expression and tumor MMP-9 secretion in MPM. We also illustrate the association between MSLN expression and local tumor invasion in MPM. These results are consistent with the fact that MSLN expressing solid tumors such as MPM, pancreatic cancer, and serous ovarian carcinoma uniformly display regionally aggressive features and dismal outcomes. We have shown our findings not only in vitro, but also in vivo. The strength of these observations is highlighted by the use of a clinically relevant orthotopic mouse model that accurately recapitulates human disease and provides an appropriate pleural tumor microenvironment to investigate tumor cell invasion and MSLN biology. Our observations are reproduced with both human and murine MSLN cells and have been verified in samples from 139 human epithelioid MPM tumors. This study provides a better understanding of the locoregional aggressiveness of MSLN expressing tumors, shows impact of MSLN on tumor MMP, and provides rationale for further clinical study of MSLN as a potential therapeutic target.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

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