



Metastasis suppressor 1 (MTSS1) expression is associated with reduced in-vivo metastasis and enhanced patient survival in lung adenocarcinoma

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Abstract

Metastasis suppressor 1 (MTSS1) has been shown to be a metastasis suppressor in a number of cancers. However, its role in lung adenocarcinoma is largely unknown. To evaluate the significance of MTSS1 expression on lung adenocarcinoma metastatic properties, the gain or loss of MTSS1 in in vivo and in vitro experiments were employed. Using an in vivo orthotopic mouse xenograft model mimicking human disease progression, stable overexpression of MTSS1 in lung adenocarcinoma cells resulted in a significant decrease in metastatic burden. Stable overexpression of MTSS1 in NCI-H1299 decreased in vitro lung adenocarcinoma invasion and migration while knockdown of MTSS1 in A549 resulted in a significant increase in cell invasion and migration. Using The Cancer Genome Atlas dataset of over 500 patient lung adenocarcinoma specimens, we demonstrated a 20% increase in 5-year survival associated with preserved intratumoral MTSS expression. MTSS1 expression in lung adenocarcinoma is associated with decreased metastatic burden, as assessed by an in vivo orthotopic model, and correlates with a 20% survival advantage at 5 years following diagnosis. In vitro data suggests MTSS1 regulates lung adenocarcinoma through augmentation of cell invasion and migration.

Keywords Lung cancer · MTSS1 · Metastasis · Adenocarcinoma

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Abbreviations

MTSS1	Metastasis suppressor 1
TCGA	The Cancer Genome Atlas
NSCLC	Non-small cell lung cancer
FACS	Fluorescence-activated cell sorting
GFP	Green fluorescent protein

Introduction

Lung cancer continues to be the most common cancer worldwide as well as the most lethal type of cancer resulting in 1.69 million deaths per year as estimated by the World Health Organization. Non-small cell lung cancer (NSCLC) represents 85% of all lung cancers with lung adenocarcinoma being the most common type [1]. The majority of patients die from NSCLC as a result of disease progression and metastasis [1]. Of patients with early stage disease who undergo lung resection for cure, 30% will go on to develop recurrent disease within 5 years [2]. Therefore, it is imperative to investigate the mechanisms of metastasis related to lung cancer disease progression.

Metastatic suppressor 1 (MTSS1) is a 755 amino acid protein which binds to actin and promotes cytoskeleton organization [3]. MTSS1 was first described by Lee and colleagues in 2002 when its transcript was found missing in metastatic bladder cancer cell lines [3]. Decreased MTSS1 expression has been shown to increase cancer cell invasion and promote metastasis in a number of cancer types including colorectal, ovarian, esophageal, prostate, hepatocellular, and bladder cancers [4–10].

To date, there is minimal data on the role of MTSS1 in NSCLC and specifically lung adenocarcinoma. Kayser and colleagues demonstrated that decreased MTSS1 expression in NSCLC tumor specimens was correlated with poor survival [11]. Previous cell culture studies suggested that overexpression of MTSS1 in lung cancer cell lines decreased cell migration and invasion in culture models, but this was not demonstrated in an animal model of this disease [12]. Currently, no animals models have investigated the role of MTSS1 in NSCLC.

This study demonstrates that in an orthotopic mouse model which mimics the pattern of human disease progression, stable overexpression of MTSS1 in lung adenocarcinoma cells results in a significant decrease in tumor burden of metastatic sites. Furthermore, our *in vitro* data demonstrates that increased MTSS1 expression is associated with decreased cell migration and invasion and vice-versa. Corroborating with our *in vitro* and *in vivo* data, we found that preservation of MTSS1 expression in primary lung adenocarcinoma tumors is associated with a 20% survival advantage using a robust dataset, The Cancer Genome Atlas (TCGA), which includes over 500 lung adenocarcinoma specimens. Collectively, these data suggest that MTSS1 expression in lung adenocarcinoma may be a useful adjunct to stratify patients at higher risk of death within 5 years of diagnosis.

Methods

Cell lines and culture conditions

Normal bronchial epithelium (NL-20) and human lung adenocarcinoma cell lines, H358, A549, and NCI-H1299 were obtained from American Type Culture Collection (ATCC, Manassas, VA) in 2015 and grown in DMEM (GE Healthcare Life Sciences, Logan, UT), supplemented with 5% Fetal Bovine Serum (Atlanta biological, Lawrenceville, GA) and 1% GlutaMAX™ (Life Technologies, Carlsbad, CA) at 37 °C humidified 5% CO₂ atmosphere incubator. Cells used for experiments were of passage 15 or less. Cell authentication and validation was performed using short tandem repeat analysis for all cell lines used in the study.

Generation of lung adenocarcinoma cells stably expressing MTSS1

NCI-H1299 cells were overexpressed with MTSS1 mCherry tagged plasmid (GeneCopoeia, Rockville, MD) using HEK293T stable transfection. The cells were put under puromycin selection pressure and then sorted using BD FACS Diva8.0.1. A549, NCI-H1299 and NCI-H1299 cells expressing high MTSS1 levels were tagged with GFP and selected with hygromycin [13].

Transfection of siRNA for knockdown of MTSS1 in lung adenocarcinoma cells

Transient silencing was accomplished using small interfering RNA targeting human MTSS1 (ID#21918, with the following sequence GGCAATTCAGAAAGTGAATT) with Lipofectamine 2000 reagent (Life Technologies, Carlsbad, CA), as previously described [14].

Cell proliferation assay

5×10^3 cells were seeded in 96-well plates and allowed to grow for 48 and 72 h. Later, cell proliferation was assessed using MTS assay (Promega, Madison, WI) as previously described [15].

Western blot

Whole cell lysates were obtained by lysing the cells in RIPA lysis buffer containing protease and phosphatase inhibitors (Pierce Biotechnology, Rockford, IL). The protein was quantified using Pierce™ BCA Protein Assay Kit, according to the manufacturer's instructions. 30 µg of protein was used for western blotting and probed with required antibodies according to the recommended dilutions. Antibodies used in this study were MTSS1 (Thermo Fisher Scientific, Waltham, MA), α-tubulin (Sigma, St. Louis, MO) and secondary antibodies HRP-conjugated mouse and rabbit (Santa Cruz Biotechnology, Inc. Dallas, TX). Secondary antibodies were used at 1:6000 dilution. Validation of the specificity of all antibodies was performed by transfecting siRNA to the specific protein of interest prior to conducting studies.

Platypus invasion and migration assays

Invasion and migration of cells were assessed using Oris™ Cell Migration Assays (Platypus Technologies, LLC, Madison, WI), according to the manufacturer's instructions. Details of the procedure are as follows: Rinse silicon stoppers with 70% ethanol and allow them to dry in the

biosafety cabinet. Using the Oris Stopper Tool, the stoppers are placed into each well and the underside of the 96-well plate inspected to ensure stoppers are fully sealed. Cells were trypsinized and counted. For migration assays, 2.5×10^4 cells were plated in 96-well plate with stoppers. After 24 h, the stoppers were removed, cells rinsed and media was added to the wells. For invasion assays, the cells were grown similarly in 50 $\mu\text{g/ml}$ collagen-coated plate. After removal of stoppers, cells were rinsed and a layer of 1.5 mg/ml neutralized collagen was added prior to adding media. Pictures were taken for analysis at 0, 24 and 48 h after the stoppers were removed. Migration and invasion areas were quantified using ImageJ Software [16].

Development of an orthotopic xenograft murine lung adenocarcinoma model

Animal experiments were performed according to the protocols approved by the IACUC at the Pennsylvania State University. Lung adenocarcinoma xenografts were created in the left lung of 6 week-old athymic nude mice (Envigo Laboratories, Frederick, MD) by injection with 60 μl of Matrigel Basement Membrane Matrix (Corning, Bedford, MA) containing 1×10^6 cells (A549-GFP, H1299-GFP and H1299-MTSS1-GFP), as previously described [17]. This animal model consistently forms a primary tumor in the left lung with subsequent generation of metastasis in mediastinal lymph nodes and contralateral lung. This pattern of metastasis mimics disease progression seen clinically. Mice were euthanized at 50 days post orthotopic injection. Necropsy was performed; internal organs (lungs, lymph nodes, adrenal, liver, and brain) were removed, compressed between two tissue culture plates and examined under microscope to count/measure metastatic deposits. ImageJ software was used to determine the percent of tumor burden area relative to that of the total organ.

Statistical analysis

Data from all experiments is represented as mean \pm standard error of mean of three separate experiments performed in triplicates. Student *t*-test was used for comparison between groups. Kaplan–Meier survival estimates were used to evaluate MTSS1 expression and patient survival and difference between groups determined by log-rank test. Results with a *p*-value of < 0.05 were considered significant. Statistical analysis was performed using Prism version 6 for MAC (GraphPad Software, Inc., La Holla, CA).

Results

Generation of an orthotopic lung adenocarcinoma mouse xenograft model which mimics human lung cancer progression

As no prior study has investigated the effect of MTSS1 expression in lung adenocarcinoma using an *in vivo* model, we utilized an *in vivo* orthotopic xenograft murine model which mimics the pathogenesis of lung adenocarcinoma seen clinically [17]. Figure 1a shows representative imaging of GFP-tagged adenocarcinoma cells forming a primary tumor in the left lung as well as metastasis to mediastinal lymph nodes and contralateral lung 10 days following injection. This pattern of metastasis accurately reflects disease progression seen clinically with patients who are diagnosed with lung adenocarcinoma. Figure 1b shows the extent of disease found at necropsy at 50 days following orthotopic injection of A549 cells. Figure 1c shows the high success rate of this orthotopic model in two lung adenocarcinoma cell lines for generation of a primary tumor and metastasis to thoracic sites as well as the development of distant sites of metastasis. With the establishment of this orthotopic, clinically relevant, *in vivo* model, we have an animal model to assess the role of MTSS1 in lung adenocarcinoma metastasis.

MTSS1 protein expression decreases in lung adenocarcinoma cell lines with more invasive characteristics and in metastatic sites compared to primary tumor *in vivo*

Figure 2a demonstrates a progressive decrease in MTSS1 protein expression in human lung adenocarcinoma cell lines with increasing invasive characteristics. Normal human bronchial epithelial cells (NL20) were found to have the highest MTSS1 protein expression and H1299 cells, which are lung adenocarcinoma cells derived from a metastatic site, have the lowest protein expression of MTSS1. Figure 2b demonstrates the same phenomenon of decreased MTSS1 protein expression in metastatic sites compared to the site of the primary tumor using the described *in vivo* orthotopic murine NSCLC model. Together, this *in vitro* data and *in vivo* data support the importance of the loss of MTSS1 expression in the development of metastasis in lung adenocarcinoma.

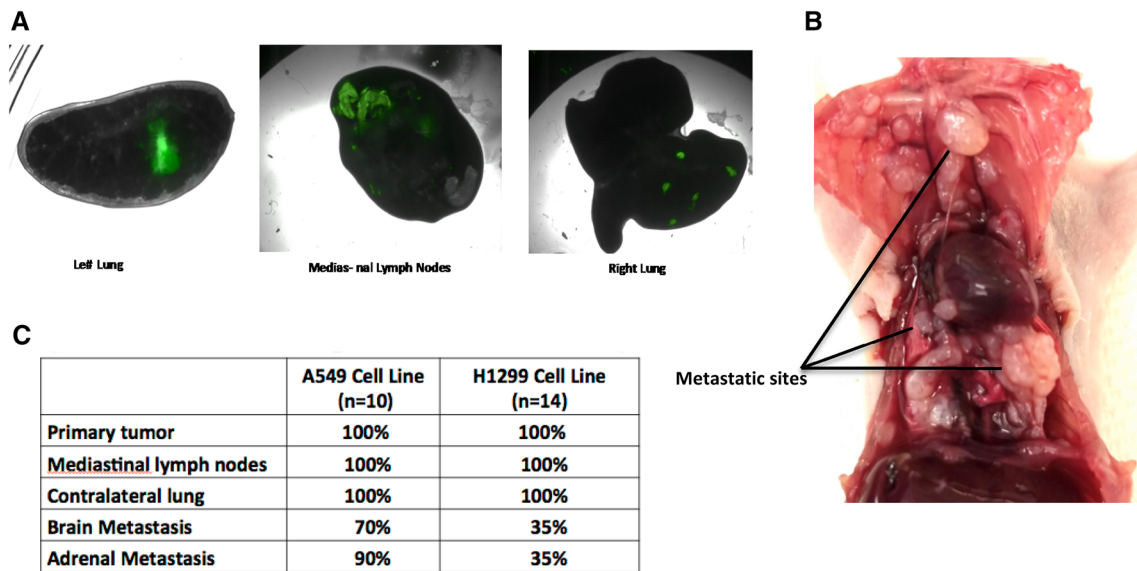
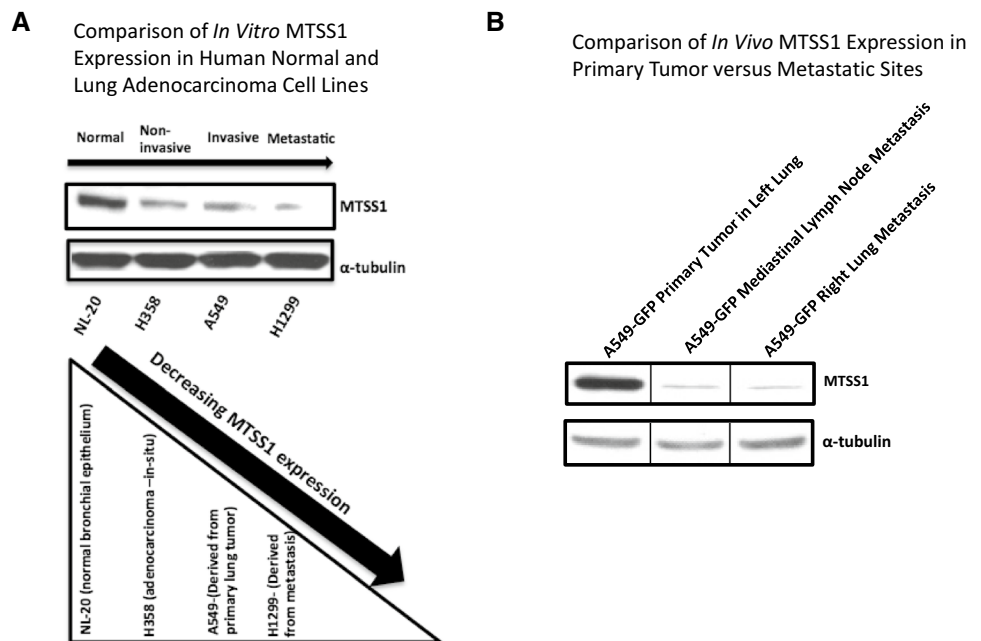


Fig. 1 **a** Representative images of whole organs at 10 days following transthoracic/orthotopic injection of GFP-tagged A549 cells (1.0×10^6 cells mixed with Matrigel to a dilution of $\times 60$). Development of a left lung primary tumor as well as mediastinal lymph node and contralateral metastasis is evident at 10 days post injection and mimics the pattern of disease spread seen clinically in patients with

lung adenocarcinoma. **b** Representative image of the diffuse disease in the left lung, mediastinal lymph nodes, and contralateral lung at 50 days following transthoracic injection of A549 adenocarcinoma cells. **c** Success of model in the development of primary tumor and metastasis in A549 and NCI-H1299 adenocarcinoma cell lines

Fig. 2 **a** MTSS1 protein expression of human normal bronchial epithelial cells (NL-20) and human lung adenocarcinoma cell lines. MTSS1 protein expression is highest in normal bronchial epithelium and progressively decreases as cells transition from adenocarcinoma-in-situ (H358) to adenocarcinoma (A549) and subsequent to metastatic lung adenocarcinoma (H1299). **b** Protein expression of MTSS1 in primary tumors compared to metastatic sites following in-vivo orthotopic injection. MTSS1 protein expression is significantly decreased in sites of metastasis (mediastinal lymph node and right lung) compared to the site of primary tumor (left lung)



Stable overexpression of MTSS1 in a murine orthotopic xenograft model inhibits metastasis

Using the above described in vivo model, we next evaluated the ability of MTSS1 to prevent lung adenocarcinoma metastasis. NCI-H1299 human lung adenocarcinoma

cells, with low endogenous MTSS1 expression, were stably transfected with mCherry red-tagged MTSS1 plasmid (Fig. 3a). Supplemental Fig. 1 illustrates selection of H1299 with high stable overexpression of MTSS1 as demonstrated by expression of the mCherry fluorophore. These cells were also stably co-expressed with GFP for the

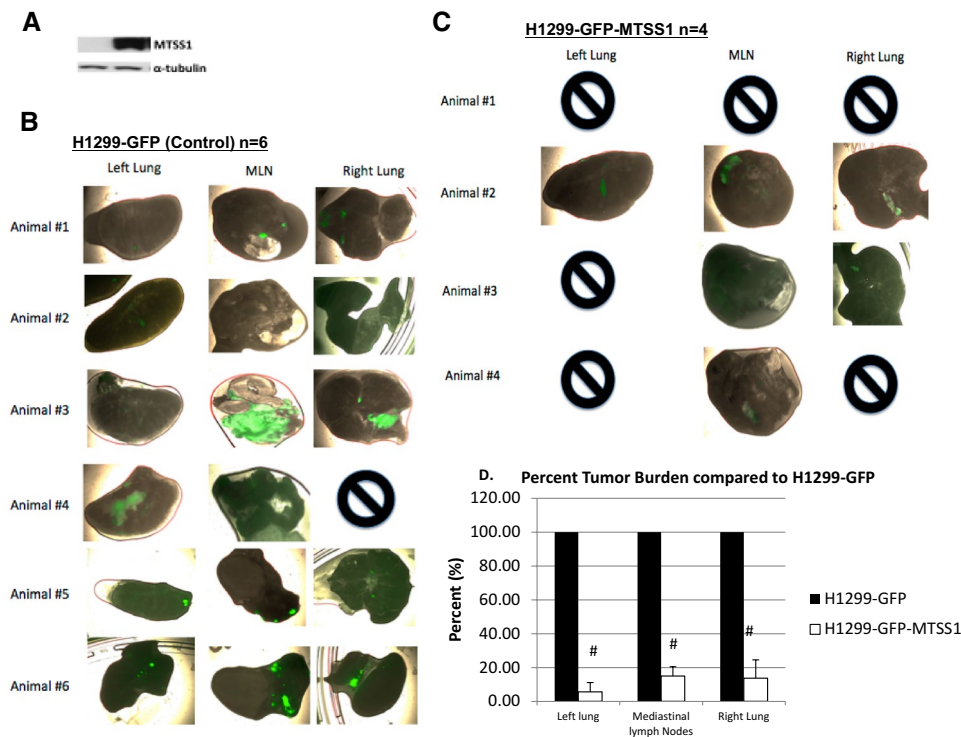


Fig. 3 **a** Western blot analysis of MTSS1 protein expression in NCI-H1299 lung adenocarcinoma cell line stably overexpressing MTSS1. **b–d** Tumor burden at 50 days following orthotopic left lung injection of human lung adenocarcinoma cells (NCI-H1299). **b** Whole organ imaging of GFP-tagged human NCI-H1299 adenocarcinoma cells (n=6) demonstrates primary tumor formation in the left lung with metastasis to mediastinal lymph node and contralateral (right) lung. **c** Whole organ imaging of GFP-tagged human NCI-H1299 cells

stably overexpressing MTSS1 (NCI-H1299-GFP-MTSS1) (n=4) demonstrates a significant decrease in primary tumor formation and metastatic tumor burden 50 days following orthotopic injection. **d** Quantification of GFP-tagged lung adenocarcinoma cells reveals a 90% decrease in primary tumor size and 85% decrease in metastatic tumor burden of H1299 cells stably overexpressing MTSS1 compared to parental H1299 adenocarcinoma cells. *MLN* mediastinal lymph nodes. #p-value < 0.05, ⊘ no detectable GFP cells in specimen

detection of metastatic sites. Figure 3b, c show the whole organ tumor burden of the primary tumor (left lung) and thoracic sites of metastasis 50 days following injection of NCI-H1299-GFP and NCI-H1299-GFP-MTSS1 stably overexpressing MTSS1 cells into athymic mice, respectively. Stable overexpression of MTSS1 resulted in a 90% decrease in tumor burden within the site of the primary tumor (left lung), 87% decrease in mediastinal lymph node metastasis, and 85% decreased in contralateral lung metastasis (Fig. 3d). Therefore, using an in vivo orthotopic model which mimics human disease progression, we have shown that MTSS1 significantly reduces metastasis in lung adenocarcinoma.

MTSS1 gene expression is downregulated in lung adenocarcinoma primary tumors and is associated with reduced survival

To correlate our in vivo findings with patient survival, MTSS1 expression in primary lung adenocarcinoma was compared to normal lung specimens found in the TCGA

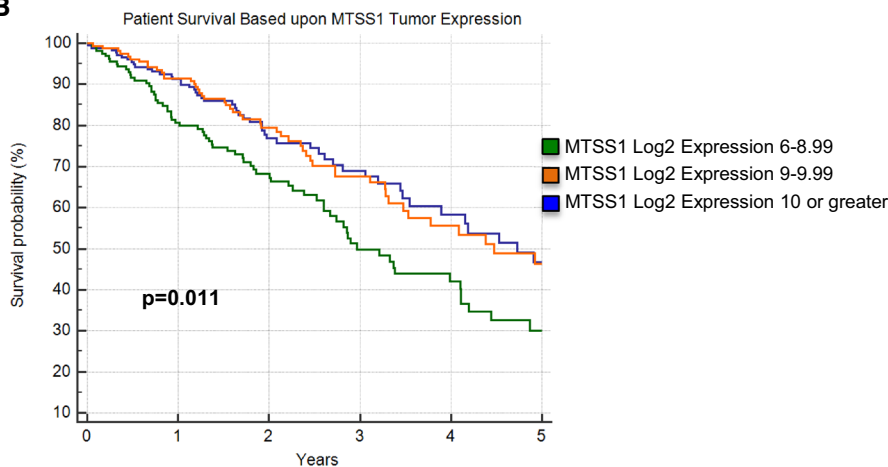
database which includes a robust sample size of greater than 500 human lung adenocarcinoma specimens. The fold change of MTSS1 expression in tumor compared to normal lung tissue was 0.68 indicating a 32% decrease of MTSS1 expression in tumors compared to normal lung (Fig. 4a). Patient MTSS1 tumor expression was then stratified into three categories based upon log MTSS1 expression (6–8.99, 9–9.99, and greater than ten) and patient survival evaluated for up to 5 years, which represents the timeframe in which lung cancer typically recurs (Fig. 4b). Comparison of log tumor MTSS1 expression of 9–9.99 versus 10 or greater did not show a significant difference in 5-year survival. However, log MTSS1 expression values of less than nine was associated with a 10% decrease in overall survival at 1 and 2 years and 20% decrease in overall survival at years 3–5 when compared to primary tumors with log MTSS1 expression values of 9 or greater. Therefore, loss of MTSS1 expression in primary tumors of patients with lung adenocarcinoma is associated with a 20% decrease in 5-year survival. This correlation of decreased MTSS1 tumoral expression and reduced patient survival suggests MTSS1 expression

Fig. 4 a MTSS1 gene expression in human lung adenocarcinoma specimens from The Cancer Genome Atlas (TCGA). Differential log expression of MTSS1 in normal lung tissue and lung adenocarcinoma primary tumor samples from The Cancer Genome Atlas Database. **b** Kaplan–Meier 5-year survival of lung adenocarcinoma patients from The Cancer Genome Atlas Database based upon tumor MTSS1 primary tumor expression

A

	Normal (n=57)		Tumor (n=514)		Tumor/Normal	
	Log2 Expression		Log2 Expression		Fold Change	p-value
MTSS1 expression	Mean	SD	Mean	SD		
	10.07	0.40	9.52	1.15	0.68	0.004

B



Number at risk

Group: MTSS1 Log2 Expression 6-8.99	162	115	69	35	23	12
Group: MTSS1 Log2 Expression 9-9.99	161	135	76	48	26	19
Group: MTSS1 Log2 Expression 10 and greater	179	145	75	48	27	20

in lung adenocarcinoma may be important for the prevention of metastasis in patients with lung adenocarcinoma and provides clinical evidence to support further investigation of MTSS1 expression in lung adenocarcinoma metastasis.

Knockdown of MTSS1 protein expression increases migration and invasion of lung adenocarcinoma cell lines

To assess the mechanism by which MTSS1 expression reduces metastasis in lung adenocarcinoma, we performed knockdown experiments using siRNA technology in a lung adenocarcinoma cell line (A549) which has moderate baseline endogenous MTSS1 expression. Transfection of the adenocarcinoma cells with siMTSS1 resulted in 98.5% knockdown of MTSS1 expression (Fig. 5a and Supplemental Fig. 2). Platypus migration and invasion assays were performed comparing siScramble transfected A549 cells to siMTSS1 transfected A549 cells at 48 h following plating. Knockdown of MTSS1 resulted in a 2.5-fold increase in cell migration (Fig. 5b). Also, knockdown of MTSS1 in lung adenocarcinoma cells yielded a 1.5-fold increase in cell invasion compared to control (siScramble) (Fig. 5c).

Investigation of the effect of knockdown of MTSS1 in lung adenocarcinoma cells revealed no change in proliferative capacity associated with decreased MTSS1 expression (Fig. 5d). Representative migration and invasion images were illustrated in Fig. 5e.

Stable overexpression of MTSS1 in lung adenocarcinoma cells results in decreased migration and invasion

Stable overexpression of MTSS1 in lung adenocarcinoma cells was associated with a 50% decrease in both migration and invasion compared to controls (NCI-H1299-GFP) (Fig. 6a, b). Stable overexpression of MTSS1 in lung adenocarcinoma cells did not affect the proliferative capacity of cells compared to controls (Fig. 6c). The platypus assay was also conducted without the presence of serum (Supplemental Fig. 3) and the decrease in cell migration associated with stable overexpression of MTSS1 in H1299 cells persisted, further supporting that the change in cell migration was not due to enhanced cell proliferation. Representative images of the platypus assay demonstrate a decrease in migration and invasion with stable overexpression of MTSS1 in

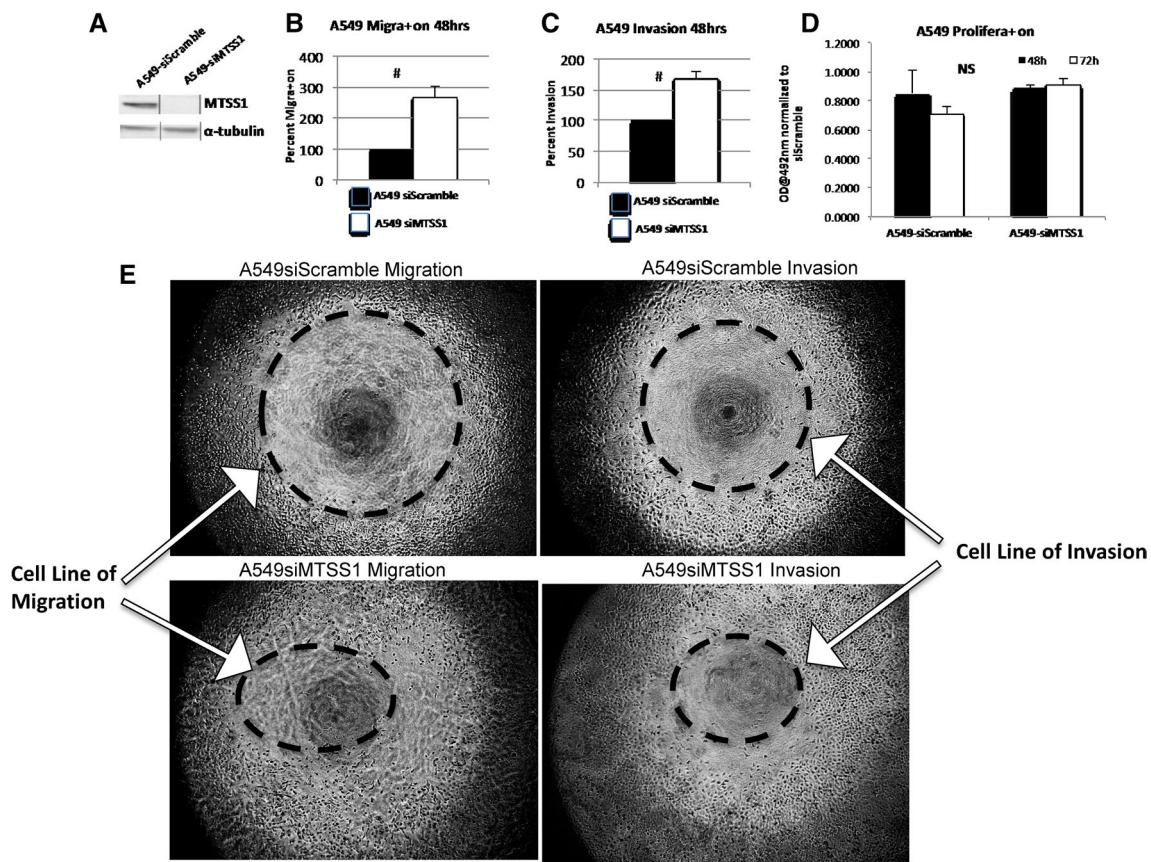


Fig. 5 **a** Western blot analysis of MTSS1 protein expression in A549 lung adenocarcinoma cell line treated with MTSS1 siRNA. Quantitative graphs representing an increase in cell migration (**5b**) and cell invasion (**5c**) associated with knockdown of MTSS1 in A549 adenocarcinoma cells. **d** Knockdown of MTSS1 in lung adenocarcinoma

cells does not affect cancer cell proliferation. **e** Platypus migration and invasion assays with representative images demonstrate a significant increase in cancer cell migration and invasion associated with knockdown of MTSS1. # p -value < 0.001, NS p -value = not significant

NCI-H1299 (H1299-GFP-MTSS1) compared to controls (H1299-GFP) (Fig. 6d).

Discussion

This study has investigated the role of MTSS1 expression in both patient survival and prevention of metastasis in lung adenocarcinoma. We have demonstrated that MTSS1 inhibits migration and invasion of lung adenocarcinoma cells in vitro and inhibits metastasis in an in vivo orthotopic model which mimics the pattern of metastasis seen clinically. Also, decreased tumor MTSS1 expression was associated with a 20% decrease in survival at years 3, 4, 5 following the diagnosis of lung adenocarcinoma. Decreased MTSS1 expression has been shown to result in an increase in metastatic potential in a number of cancers including pancreatic [18], breast [19], ovarian [7], hepatocellular [10], head and neck [20], and colorectal [4].

We have previously demonstrated that patients with early stage NSCLC who have no evidence of lymph node metastasis and undergo surgical resection for curative intent have a 30% cancer recurrence rate at 5 years [2]. Therefore our evaluation of MTSS1 expression in lung adenocarcinoma tumor specimens using the TCGA cohort focused on survival 5 years after diagnosis. Evaluation of the Kaplan–Meier survival estimates for this large patient population suggests that decreased MTSS1 expression in patient tumors is a poor prognostic indicator. Previous work by Kayser and colleagues support our findings that down-regulation of MTSS1 in early non-small cell lung cancer tumors is associated with poor survival [11]. This evidence suggests that intratumoral MTSS1 expression may enable further stratification of patients at risk of lung cancer recurrence. Reduced intratumoral MTSS1 expression may support the use of additional therapies, such as chemotherapy or targeted therapy to lessen the risk of recurrent disease. These strong associations of MTSS1 expression and lung cancer survival further support future efforts focusing on

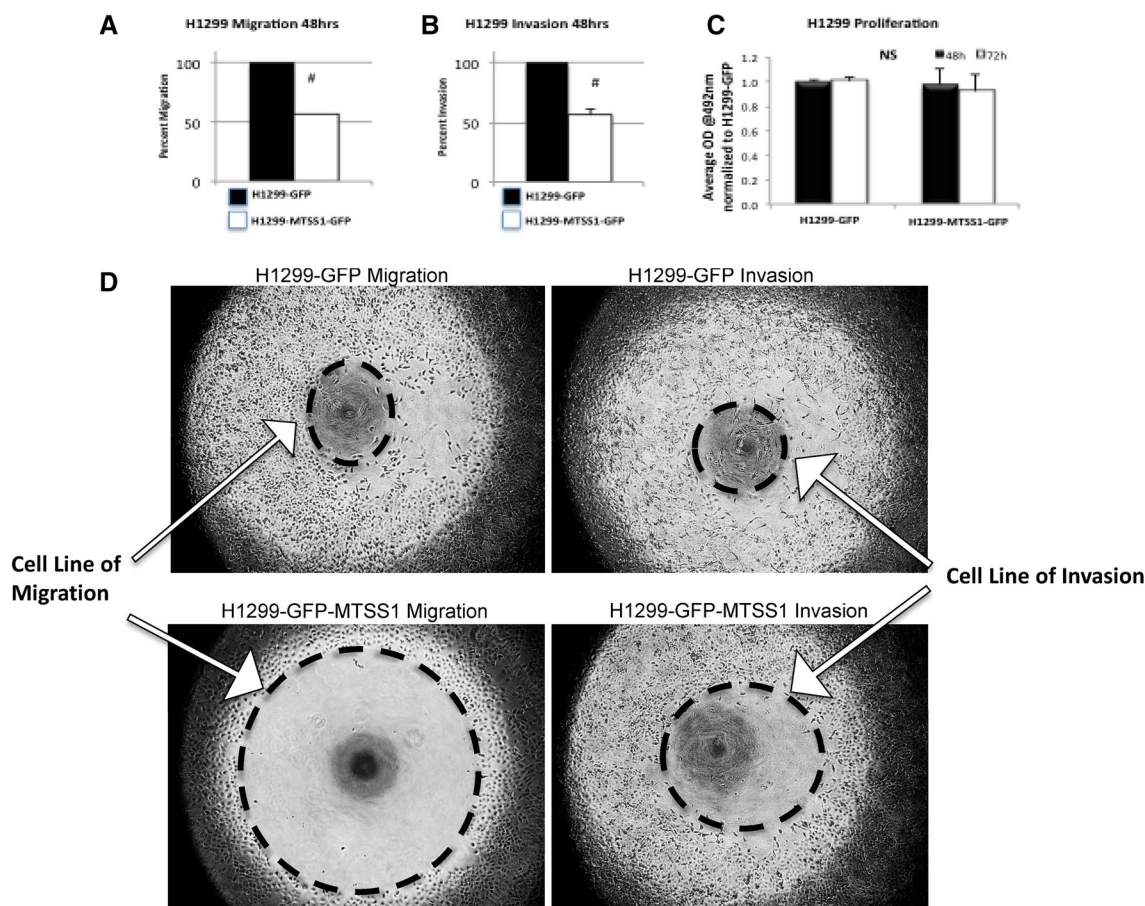


Fig. 6 Quantitative graphs representing a decrease in cell migration (**5a**) and cell invasion (**5b**) associated with overexpression of MTSS1 in NCI-H1299 adenocarcinoma cells. **c** Stable overexpression of MTSS1 in NCI-H1299 lung adenocarcinoma cells does not affect

cancer cell proliferation. **e** Platypus migration and invasion assays with representative images demonstrate a significant decrease in cancer cell migration and invasion associated with stable overexpression of MTSS1. #*p*-value < 0.001, NS *p*-value = not significant

the mechanisms which regulate MTSS1 expression in lung adenocarcinoma with the intention of developing targetable therapies aimed at preserving MTSS1 expression.

Little is known about the mechanisms by which MTSS1 regulates lung adenocarcinoma metastasis. Previous work in urothelial cancer has demonstrated that DNA methyltransferase 3B (DNMT3B) and histone deacetylase (HDAC) 1/2 bind to the MTSS1 promoter thereby epigenetically silencing MTSS1 transcription [21]. Silencing of the MTSS1 promoter increased cell migration and invasion and led to the induction of epithelial-mesenchymal transition as supported by decreased E-cadherin expression and increased vimentin expression. Dawson et al. have demonstrated that MTSS1 is known to activate the small GTPase, Rac1, which is a key regulator of the actin cytoskeleton and important mediator of cell-to-cell contacts. Activation of Rac1 by MTSS1 stabilized cell-to-cell contacts and inhibited cell scattering. In addition, MTSS1 inhibition resulted in cell disaggregation [22]. Also, upregulation of

a number of microRNAs including miR-29B, miR-135A, and miR-182 have been shown to inhibit transcription of MTSS1 promoting enhanced cancer cell invasion and migration [10, 23–25]. All these data gave us an impetus to evaluate the significance of MTSS1 in lung adenocarcinoma metastasis.

In summary, we have demonstrated that MTSS1 reduces metastasis in an in vivo orthotopic animal model which mimics lung adenocarcinoma progression observed clinically. Preservation of MTSS1 intratumoral expression in patients with lung adenocarcinoma is associated with a 20% increase in overall survival at 5 years following diagnosis. In vitro studies suggest that MTSS1 regulates both cell migration and invasion in lung adenocarcinoma. Future directions will further evaluate the mechanisms related to MTSS1 regulation in lung adenocarcinoma metastasis.

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Compliance with ethical standards

Conflict of interest The authors have declared that no conflicts of interest exist.

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