

TMEM139 prevents NSCLC metastasis by inhibiting lysosomal degradation of E-cadherin

Shuai Zhang¹  | Yunlong He² | Qijia Xuan³ | Xiaodong Ling⁴ | Kaiya Men¹ | Xu Zhao¹ | Dinglong Xue¹ | Ling Li¹ | Yingying Zhang⁵

¹Department of Medical Oncology, Harbin Medical University Cancer Hospital, Harbin, China

²Department of Radiation Oncology, Harbin Medical University Cancer Hospital, Harbin, China

³Department of Medical Oncology, The Fourth Affiliated Hospital Zhejiang University School of Medicine, Yiwu, China

⁴Department of Thoracic Surgery, Harbin Medical University Cancer Hospital, Harbin, China

⁵Department of Medical Oncology, Hu Lun Bei Er Ren Min Hospital, Hulunbuir, China

Correspondence

Shuai Zhang, Department of Medical Oncology, Harbin Medical University Cancer Hospital, 150 Haping Road, Nangang District, Harbin, Heilongjiang Province 150040, China.
Email: zshuai@hrbmu.edu.cn

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Abstract

Non-small-cell lung cancer (NSCLC) accounts for approximately 85% of all lung cancer cases and has the highest mortality rate among all solid tumors. It is characterized by early metastasis, and investigations of the molecular mechanisms underlying the progression and metastasis of NSCLC are urgently needed for the development of therapeutic targets. Here, we report that the transmembrane protein TMEM139 is significantly downregulated in NSCLC and that reduced expression of TMEM139 is correlated with a poor prognosis in NSCLC patients. Mechanistically, we found that TMEM139 directly interacts with E-cadherin at the plasma membrane and at focal adhesion sites. Moreover, TMEM139 can prevent the lysosomal degradation of E-cadherin, which inhibits epithelial-mesenchymal transition, migration and invasion of NSCLC cells both in vitro and in vivo. Our study not only expands our understanding of NSCLC metastasis but also provides a foundation to develop novel therapeutic strategies.

KEYWORDS

E-cadherin, epithelial-mesenchymal transition, lysosomal degradation, non-small-cell lung cancer, TMEM139

1 | INTRODUCTION

Lung cancer is one of the most common cancers in men and women, and is the leading cause of cancer-related death (approximately 25% of all cancer deaths) worldwide.^{1,2} Most cases of lung cancer are non-small-cell lung cancer (NSCLC) (approximately 85%), which can be further classified as adenocarcinoma, squamous cell carcinoma,

or large cell carcinoma.³ Although multiple strategies, including surgery, chemotherapy, radiotherapy, and immunotherapy, have been applied in the clinical treatment of NSCLC, the 5-year overall survival (OS) rate of NSCLC patients at all stages is only 16%.^{4,5} Thus, studies that investigate the molecular mechanisms underlying the progression and metastasis of NSCLC would be valuable for development of novel therapeutic options for lung cancer in the clinic.

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Epithelial-mesenchymal transition (EMT) is a phenotypic switching process that induces a loss of epithelial features and a gain of mesenchymal features, including enhanced migration and invasion capacities.⁶ EMT of tumor cells plays a crucial role in facilitating tumor progression and metastasis.⁷ It is well known that decreased expression of E-cadherin, a cell adhesion molecule located at the plasma membrane, is important for initiation of the EMT process.⁸ Several transcription factors, such as zinc finger E-box-binding transcription factor 1 (ZEB1), transcription factor 3 (TCF3), and Krüppel-like factor 8 (KLF8), have been reported to inhibit the expression of E-cadherin by binding to its promoter.^{7,9} In addition to transcriptional regulation, the expression of E-cadherin can also be regulated at the posttranscriptional level. It has been shown that lysosome-dependent cellular degradation of E-cadherin also promotes EMT and tumor metastasis.^{10,11} However, the detailed molecular mechanisms involved in lysosome-dependent E-cadherin degradation are still largely unclear and need to be further investigated.

Transmembrane protein 139 (TMEM139) is a novel transmembrane protein, the expression and function of which have not been studied in detail. It has been predicted that TMEM139 may be located at the plasma membrane and in focal adhesion sites.¹² Recently, a bioinformatic study that searched for key biomarkers and potential molecular mechanisms in lung cancer identified TMEM139 as one of the differentially expressed genes in lung cancer.¹³ In this study, we aimed to investigate whether TMEM139 is involved in NSCLC progression and metastasis. Our study showed that TMEM139 was significantly downregulated in NSCLC and that reduced expression of TMEM139 was correlated with a poor prognosis in NSCLC patients. Mechanistically, we found that TMEM139 directly interacts with E-cadherin and prevents its lysosomal degradation, which inhibits NSCLC cell EMT, migration and invasion both *in vitro* and *in vivo*.

2 | MATERIALS AND METHODS

2.1 | Patient samples

One hundred and seven clinical samples were taken from NSCLC patients who underwent surgical treatment at the Cancer Hospital of Harbin Medical University from January 2012 to December 2013. Before sample collection, none of the patients received any anticancer treatments. All experiments were conducted in accordance with the relevant guidelines and regulations of Harbin Medical University, and the research was approved by the Ethics Committee of Harbin Medical University. Written informed consent was obtained from each patient.

2.2 | Cell lines and cell culture

A549 and H1299 human lung adenocarcinoma cell lines were obtained from the American Type Culture Collection (ATCC). Both cell lines were cultured in RPMI 1640 (Gibco) supplemented with 10%

fetal bovine serum (Gibco), 100 µg/ml streptomycin, and 100 units/ml penicillin (Invitrogen). Cells were maintained in a humidified 5% CO₂ chamber at 37°C.

2.3 | Immunohistochemistry and assessment

TMEM139 expression was evaluated via immunohistochemistry. Surgical tumor specimens from NSCLC patients were embedded in paraffin. Immunohistochemical staining was performed with anti-human TMEM139 antibody (Thermo Fisher PA5-57898, 1:100). Images were taken with an optical microscope (Zeiss). Immunostaining analysis was carried out in a double-blind manner by two experienced pathologists. Five images in independent fields of view were taken for each sample. TMEM139 expression was assessed using the Immuno-Reactive-Score (IRS) system, which is based on the intensity of anti-TMEM139 immunostaining (0–3, Figure S1A), and the positive cells proportion score (0–4). To analyze the survival curve of patients with high and low TMEM139 expression, an IRS score of ≥6 was used to define tumors with high expression of TMEM139, and an IRS score of <6 was used to indicate low expression (Table 1).

2.4 | Confocal microscopy

A549 cells were plated on coverslips overnight and transfected with GFP-tagged TMEM139 the next morning. After 2 days, the cells were washed with PBS, fixed for 0.5 h with 4% PFA in PBS, and then washed three times with PBST. For E-cadherin staining, the cells were permeabilized with 0.1% Triton X-100, blocked and incubated with anti-E-cadherin antibody (1:100) in 10% FBS in PBST for 2 h at RT. Cells were then washed with PBST and incubated with Alexa Fluor 546-conjugated secondary Ab (Invitrogen) in 10% FBS in PBST for 1 h. The nuclei were stained with DAPI (Invitrogen).

2.5 | Co-IP

HEK-293T cells were transfected with Flag-TMEM139 and GFP-E-cadherin using polyethylenimine according to the manufacturer's protocol. After 36 h, cells were collected and resuspended in lysis buffer. Extracts were immunoprecipitated with anti-Flag beads and then used for immunoblot analysis. For IP of endogenous TMEM139, A549 and H1299 cells were directly resuspended in lysis buffer, and proteins were immunoprecipitated from extracts with anti-TMEM139 antibody (1:200) and assessed via immunoblot analysis.

2.6 | Quantitative real-time PCR

Total RNA was extracted from HEK-293T, A549, and H1299 lung cancer cells using TRIzol reagent (Invitrogen). DNA was then reverse

TABLE 1 Association between TMEM139 expression and clinicopathological characteristics in 107 NSCLC patients

Variable	All patients (n = 107)	TMEM139 expression		P
		High (n = 35) (%)	Low (n = 72) (%)	
Gender				
Male	63	24 (38.1)	39 (61.9)	0.155
Female	44	11 (25.0)	33 (75.0)	
Age (years)				
<60	59	23 (38.9)	36 (61.0)	0.125
≥60	48	12 (25.0)	36 (75.0)	
pTumor size				
T1-2	82	28 (34.1)	54 (65.9)	0.566
T3-4	25	7 (28.0)	18 (72.0)	
Lymph node metastasis				
N0-1	47	17 (36.1)	30 (63.8)	0.500
N2	60	18 (30.0)	42 (70.0)	
pTNM stage				
I-II	41	16 (39.0)	25 (61.0)	0.273
III	66	19 (28.7)	47 (71.2)	
Histology type				
Adenocarcinoma	69	21 (30.4)	48 (69.6)	0.499
Squamous	38	14 (36.8)	24 (63.2)	

Abbreviations: NSCLC, non-small-cell lung cancer; pT, pathological tumor; pTNM, pathological tumor node metastasis.

transcribed using Moloney MLV reverse transcriptase (Invitrogen). Quantitative PCR was performed using SYBR Premix Ex Taq (Takara) according to the manufacturer's protocol. The expression of human TMEM139 and E-cadherin was normalized to that of human GAPDH. All primers were synthesized by Sangon Biotech as follows: TMEM139-F 5'-GCTTTGGTCACCTGATGAT-3' and TMEM139-R 5'-TGGGGTAGGTACCAAATGT-3'; E-cadherin-F 5'-TGCCAACTG GCTGGAGATTA-3' and E-cadherin-R 5'-AGTGTCCCTGTTCCAG TAGC-3'.

2.7 | Western blotting

Cells were lysed in RIPA protein lysis buffer for 40 min to obtain total proteins, and a BCA kit was used to quantify the protein concentration. Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE, 10%) was used to separate 40 µg of protein, and the proteins were transferred to a PVDF membrane. Then, the membranes were blocked with 5% nonfat milk for 1 h at room temperature, followed by incubation with diluted primary antibodies overnight at 4°C and subsequent incubation with secondary antibodies for 1 h at room temperature. The primary antibodies specific to human E-cadherin, vimentin, and GAPDH have been described previously¹⁴; the primary antibody against human TMEM139 was obtained from Thermo Fisher (PA5-57898). Mouse anti-rabbit IgG-HRP (CST) and goat anti-mouse IgG-HRP (Santa Cruz Biotechnology) were used as the secondary antibodies.

2.8 | Wound-healing assays

For wound-healing assays, cells were plated into six-well dishes and cultured to nearly 100% confluence in EC medium. Subsequently, confluent cell monolayers were scraped with plastic pipettes to manually create a cell-free mark. The cultures were washed with phosphate-buffered saline (PBS) after scratching and incubated for 48 h. The scratched wounds were observed and captured with a bright-field microscope at 0, 24, and 48 h. The rate of wound healing (closure) was analyzed to objectively assess cell migration and proliferation.

2.9 | Transwell invasion assays

The cell migratory ability was assessed using Transwell assays. In short, 24-well plates were filled with Transwell cell culture chambers (pore size, 6.5 mm) and 60 µl of a 1:3 mixture of Matrigel and serum-free RPMI 1640 was used to coat the membrane to create a matrix barrier. Cell suspensions from each group (1×10^5 cells/ml) were seeded into the upper Transwell chamber and 600 µl medium with 10% FBS was placed into the lower chamber. After incubation for 24 or 48 h, cotton swabs were used to erase the noninvasive or nonmigratory cells from the upper chambers. The migrated cells in the lower chambers were fixed and stained with 4% paraformaldehyde and 0.5% crystal violet. Migrated cells were then imaged using an inverted microscope and counted in five random fields.

2.10 | Xenograft models

BALB/C nude mice (6 weeks old) were given an intravenous injection of 2×10^6 A549 cells overexpressing either TMEM139 or vector. After 35 days, the mice were euthanized, the metastatic foci were counted, and macroscopic pictures of the lungs were taken.

2.11 | Statistical analysis

Statistical analysis was performed with GraphPad Prism software. All data are expressed as the means \pm SEM. Student's *t*-test and Spearman's *r* correlation were used as appropriate to assess statistically significant differences. $P < 0.05$ indicated statistically significant results.

3 | RESULTS

3.1 | Reduced expression of TMEM139 is correlated with a poor prognosis in NSCLC patients

To examine whether TMEM139 is involved in NSCLC progression and metastasis, we first tested the expression of TMEM139 in clinical samples from NSCLC patients. As shown in Figure 1A,B, the mRNA and protein levels of TMEM139 were significantly downregulated in NSCLC tumor samples compared with adjacent normal lung tissues. Moreover, immunohistochemical analysis confirmed that the protein levels of TMEM139 were markedly reduced in the two main histological subtypes of NSCLC tumor samples (adenocarcinoma and squamous cell carcinoma) compared with adjacent nontumor lung tissues (Figure 1C). To further determine the clinical relevance of TMEM139 expression in lung cancer progression, we analyzed the survival curve of patients with high and low TMEM139 expression. As shown in Figure 1D,E, patients with high level TMEM139 expression have longer OS and disease-free survival (DFS). Moreover, Kaplan–Meier analysis from a public database (<http://kmplot.com/analysis/index.php?p=service&cancer=lung>) also showed a similar result (Figure S1B), that a high TMEM139 transcript level is correlated with longer OS and progression-free survival (PFS) in lung cancer patients.

Taken together, these results suggest that the expression of TMEM139 is reduced in NSCLC and that lower expression of TMEM139 is clinically correlated with poor prognosis in lung cancer patients.

3.2 | TMEM139 inhibits the migration and invasion of lung cancer cells

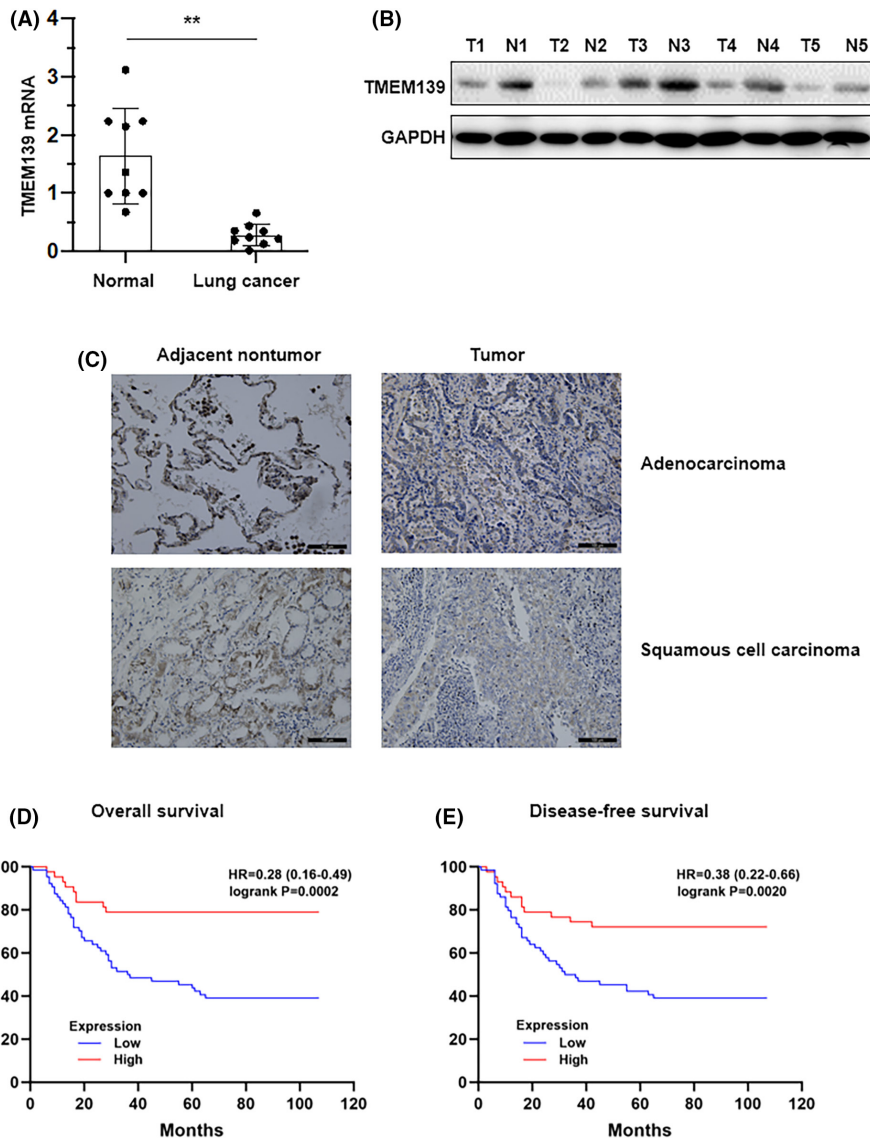
Since lower expression of TMEM139 is correlated with poor prognosis in lung cancer patients, we next investigated whether TMEM139 regulates EMT progression and inhibits the metastatic potential of

NSCLC. As TMEM139 is downregulated in NSCLC tumor samples, we first established cell lines stably expressing either vector or Flag-TMEM139 using the NSCLC cell lines A549 and H1299, and examined changes in EMT markers. As shown in Figure 2A, TMEM139 overexpression increased the expression of the epithelial marker E-cadherin and reduced the expression of the mesenchymal marker vimentin in both the A549 and H1299 cell lines. In addition to reduced E-cadherin expression, the EMT process is always accompanied by increased expression of other proteins, such as type I collagen and matrix metalloproteinases (MMPs), which are involved in regulation of cell-cell adhesion and extracellular matrix degradation.^{15–17} We confirmed that TMEM139 overexpression decreased the expression of type I collagen, MMP2, and MMP9 (Figure 2B). In addition, we also further tested whether TMEM139 inhibits the EMT-like phenotype during EMT process, as shown in Figure S2A. TMEM139 overexpression inhibited the TGF- β 1-induced EMT phenotype of the A549 cell line in vitro. These results confirm that TMEM139 inhibits EMT progression in NSCLC. To further verify the effect of TMEM139 on the migration and invasion capacities of NSCLC cell lines in vitro, we performed Transwell migratory and Matrigel invasion assays. As shown in Figure 2C,D, overexpression of TMEM139 inhibited A549 cell migration compared with vector control. Additionally, TMEM139 overexpression prevented A549 cell migration in vitro (Figure 2E,F). Collectively, our results show that TMEM139 inhibits EMT and the metastatic potential of lung cancer cells.

3.3 | TMEM139 promotes the lysosomal degradation of E-cadherin

Loss of E-cadherin expression is essential for EMT, and TMEM139 overexpression increased the expression of E-cadherin. We next investigated whether TMEM139 can directly regulate the expression of E-cadherin. As shown in Figure 3A–C, TMEM139 overexpression indeed increased the protein expression of E-cadherin in a concentration-dependent manner but had no effect on the mRNA expression of E-cadherin. This suggested that TMEM139 may regulate the expression of E-cadherin at the posttranscriptional level. Previous studies have suggested that the protein level of E-cadherin can also be regulated by lysosomal degradation.^{11,18–22} As shown in Figure 3D,E, treatment of A549 cells with the protein synthesis inhibitor cycloheximide (CHX) indeed induced the degradation and decreased the expression of E-cadherin. Moreover, we confirmed that inhibition of lysosome function by chloroquine (CQ) delayed the degradation of E-cadherin in A549 cells, while inhibition of proteasome-dependent degradation via MG132 had no effect on the expression of E-cadherin (Figure 3D,E). As shown in Figure 3F,G, TMEM139 overexpression prevented the degradation of E-cadherin but had no effect on the mRNA expression of E-cadherin. More importantly, the protein synthesis inhibitor CHX also induced decreased expression of TMEM139, similar to E-cadherin. Therefore, our results suggest that TMEM139 might increase E-cadherin protein expression by inhibiting its degradation.

FIGURE 1 TMEM139 is expressed at low levels in NSCLC and correlated with poor prognosis. (A) Relative mRNA level of TMEM139 in NSCLC tumor samples and adjacent normal lung tissues ($n = 9$). (B) Western blotting analysis of TMEM139 protein expression in NSCLC tumor samples and adjacent normal lung tissues ($n = 5$). (C) IHC analysis of TMEM139 expression in both adenocarcinoma (top) and squamous cell carcinoma (down) tumor samples and adjacent nontumor lung tissues. (D and E) Kaplan–Meier analysis of overall survival (OS) and disease-free survival (DFS) in lung cancer patients with low or high expression of TMEM139. $**P < 0.05$, mean \pm SEM



3.4 | TMEM139 interacts with E-cadherin

We next investigated how TMEM139 prevents the degradation of E-cadherin in A549 cells. As shown in Figure 3F, TMEM139 and E-cadherin were degraded in a similar manner after CHX treatment, indicating that TMEM-139 may connect with E-cadherin and that the TMEM139-E-cadherin complex is degraded together. Indeed, it has been predicted that TMEM139 may be located at the plasma membrane and in focal adhesion sites,¹² whose location is similar to that of E-cadherin. We first tested the intracellular location of TMEM139 and E-cadherin. As shown in Figures 4A and S3A, both TMEM139 and E-cadherin were located at the plasma membrane and were rich in focal adhesion sites of A549 cells. After confirming that both TMEM139 and E-cadherin share the same intracellular location, we hypothesized that TMEM139 may inhibit E-cadherin degradation by interacting with E-cadherin directly. Next, we performed coimmunoprecipitation (co-IP) experiments to test whether TMEM139 can interact with E-cadherin directly. As shown in Figure 4B, overexpressed TMEM139 strongly bound to overexpressed E-cadherin

in human embryonic kidney T (HEK-293T) cells. Moreover, endogenous TMEM139 also interacted with endogenous E-cadherin in both A549 and H1299 cell lines (Figure 4C). Collectively, these results suggest that TMEM139 directly interacts with E-cadherin and prevents its lysosomal degradation.

3.5 | TMEM139 decreases the metastatic potential of lung cancer cells in vivo

To determine whether TMEM139 is capable of suppressing NSCLC metastasis in vivo, TMEM139-overexpressing or control A549 cells were injected into immunodeficient mice to evaluate their metastatic colonization capacity in a tail vein assay. After 5 weeks, we observed a significantly decreased number of lung metastatic nodules in TMEM139-injected mice, which was confirmed by hematoxylin and eosin staining of lung sections (Figure 5A,B). The expression of TMEM139 was confirmed by immunoblotting analysis of tumors from both TMEM139-overexpressing and control A549

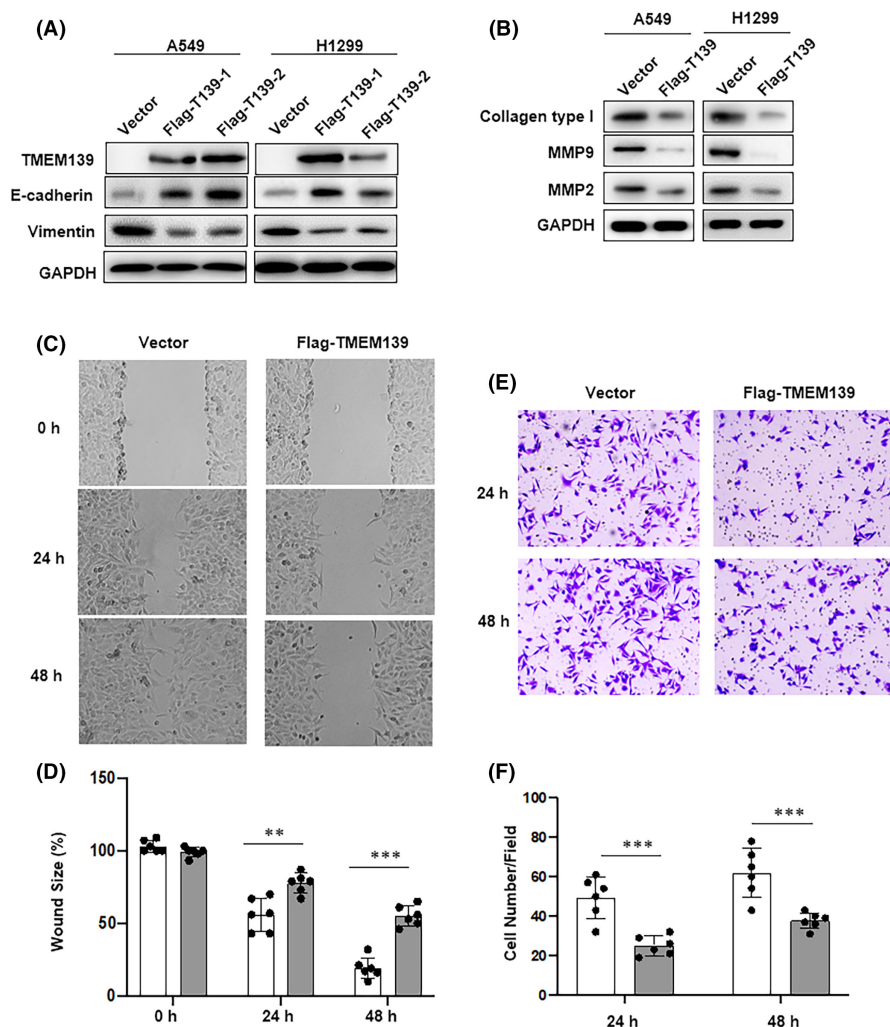


FIGURE 2 TMEM139 inhibits EMT in lung cancer cells. Stable expression of either vector or Flag-TMEM139 in A549 and H1299 cells via a lentiviral system. (A) Western blotting analysis of TMEM139, E-cadherin, Vimentin, and GAPDH protein expression in A549 and H1299 cells stably expressing vector or Flag-TMEM139. (B) Western blotting analysis of type I collagen, MMP2, MMP9, and GAPDH protein expression in A549 and H1299 cells stably expressing vector or Flag-TMEM139. (C and D) The migration ability of A549 cells stably expressing vector or Flag-TMEM139 was examined in a wound healing assay at 24 or 48 h. (E and F) Invasion assays of A549 cells stably expressing vector or Flag-TMEM139 were performed after 24 or 48 h. ** $P < 0.05$, *** $P < 0.01$, mean \pm SEM

cells (Figure 5C). Moreover, overexpression of TMEM139 indeed corresponded with an increase in E-cadherin protein expression but had no effect on E-cadherin mRNA expression (Figure 5C,D). Taken together, these results confirm that TMEM139 is a potent metastasis suppressor and can decrease the metastatic potential of lung cancer cells *in vivo*.

4 | DISCUSSION

TMEM139 is a novel transmembrane protein, the expression and function of which has not been studied in detail. Although one study suggested that TMEM139 is one of the differentially expressed genes in lung cancer,¹³ the role of TMEM139 in NSCLC progression and metastasis remains unclear.

In this study, we found that TMEM139 colocalized with E-cadherin at the plasma membrane and in focal adhesion sites of lung cancer cells. Moreover, TMEM139 can prevent the lysosomal degradation of E-cadherin, which may be achieved through its interaction with E-cadherin. However, the exact mechanism underlying how TMEM139-E-cadherin prevents the lysosomal degradation of E-cadherin in lung cancer cells remains unclear.

In this study, we found that TMEM139 was significantly down-regulated in NSCLC and that reduced expression of TMEM139 facilitated the E-cadherin degradation and promoted EMT in NSCLC. Through analysis of microarray data, two previous studies have indicated that TMEM139 is one of the downregulated genes in bladder cancer and thyroid cancer samples.^{23,24} However, the role of TMEM139 in the progression of bladder cancer and thyroid cancer has not been investigated. Whether TMEM139 can also regulate EMT in bladder cancer and thyroid cancer cells through its interaction with E-cadherin needs to be further studied in the future. In addition, although E-cadherin has been known as a key mediator of cell adhesion to prevent tumor cell migration and invasion, recent studies also suggested that E-cadherin may promote invasive and metastatic behavior in several cancers, especially in late-stage cancers.²⁵⁻²⁷ For example, E-cadherin has been shown to act as a survival factor to promote metastasis in diverse models of invasive ductal carcinomas by limiting reactive oxygen-mediated apoptosis.²⁸ Whether TMEM139-E-cadherin interaction can also facilitate cancer metastasis in other types of cancers needs to be considered and investigated.

A previous study suggested that smoking induces small airway epithelial epigenetic changes that may modulate the expression

FIGURE 3 TMEM139 prevents the lysosomal degradation of E-cadherin. (A–C) A549 cells were transfected with the indicated concentrations of Flag-TMEM139 for 24 h. (A) Western blotting analysis of TMEM139, E-cadherin, and GAPDH protein expression. (B and C) qPCR analysis of TMEM139 and E-cadherin mRNA expression. (D and E) A549 cells were treated with CQ (100 μ M) (D) or MG132 (10 μ M) (E) for 1 h and then treated with CHX (20 μ M) for the indicated times. Western blotting analysis of E-cadherin and GAPDH protein expression. (F and G) A549 cells stably expressing vector or Flag-TMEM139 were treated with CHX (20 μ M) for the indicated times. Western blotting analysis of TMEM139, E-cadherin, and GAPDH protein expression (F) and qPCR analysis of E-cadherin mRNA expression (G)

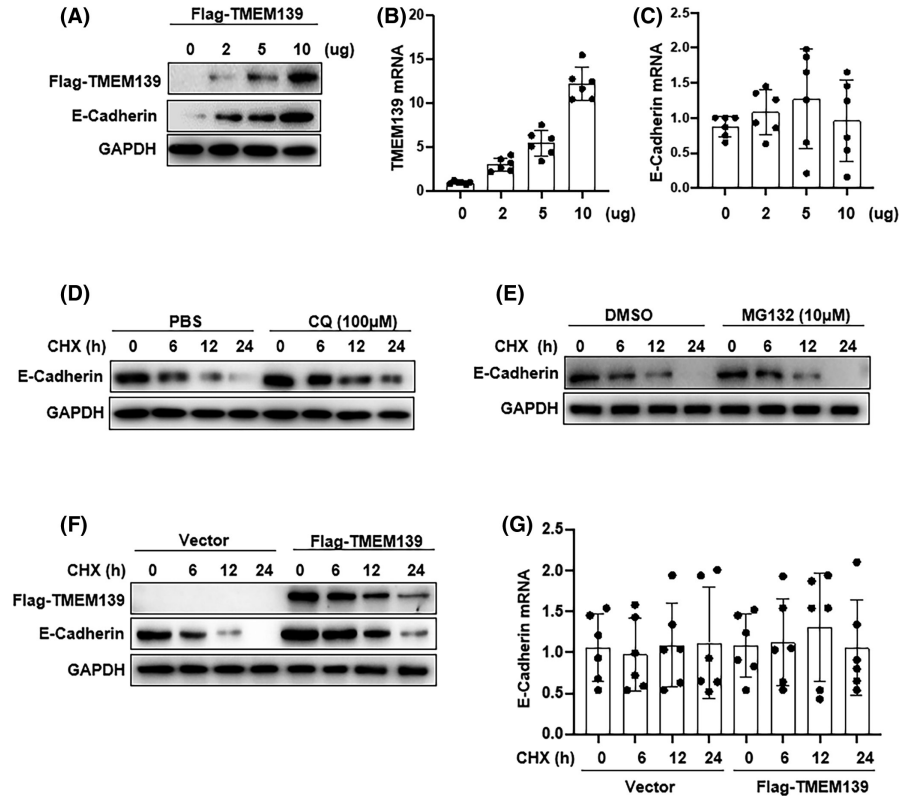
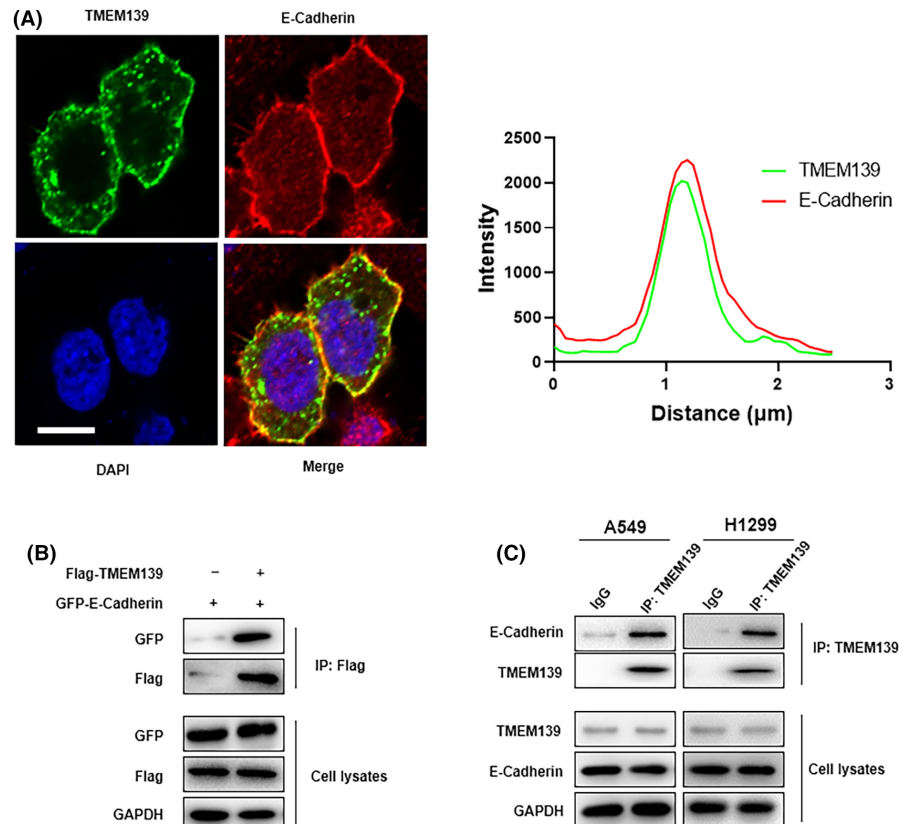


FIGURE 4 TMEM139 interacts with E-cadherin. (A) A549 cells were transfected with TMEM139-GFP for 48 h and stained with E-cadherin primary antibody (rhodamine-secondary antibody). Confocal analysis of the cellular localization of TMEM139-GFP and endogenous E-cadherin. (B) 293T cells were cotransfected with a plasmid encoding Flag-TMEM139 and GFP-E-cadherin. Whole cell extracts were immunoprecipitated with anti-flag antibody. (C) Whole cell lysates were extracted from A549 or H1299 cells and then immunoprecipitated with anti-TMEM-139 antibody or an equal amount of mouse IgG. Western blotting was performed for analysis of proteins in both the immunoprecipitation and cell lysates



of various genes.²⁹ Among these genes, TMEM139 is one of the hypermethylated genes in smokers compared with nonsmokers.²⁹ Since cigarette smoking is the number one risk factor for lung cancer and small airway epithelium is the first site of

smoking-induced lung pathology,³⁰ whether smoking-induced DNA methylation of the TMEM139 gene is involved in smoking-related lung cancer progression needs to be further investigated in the future.

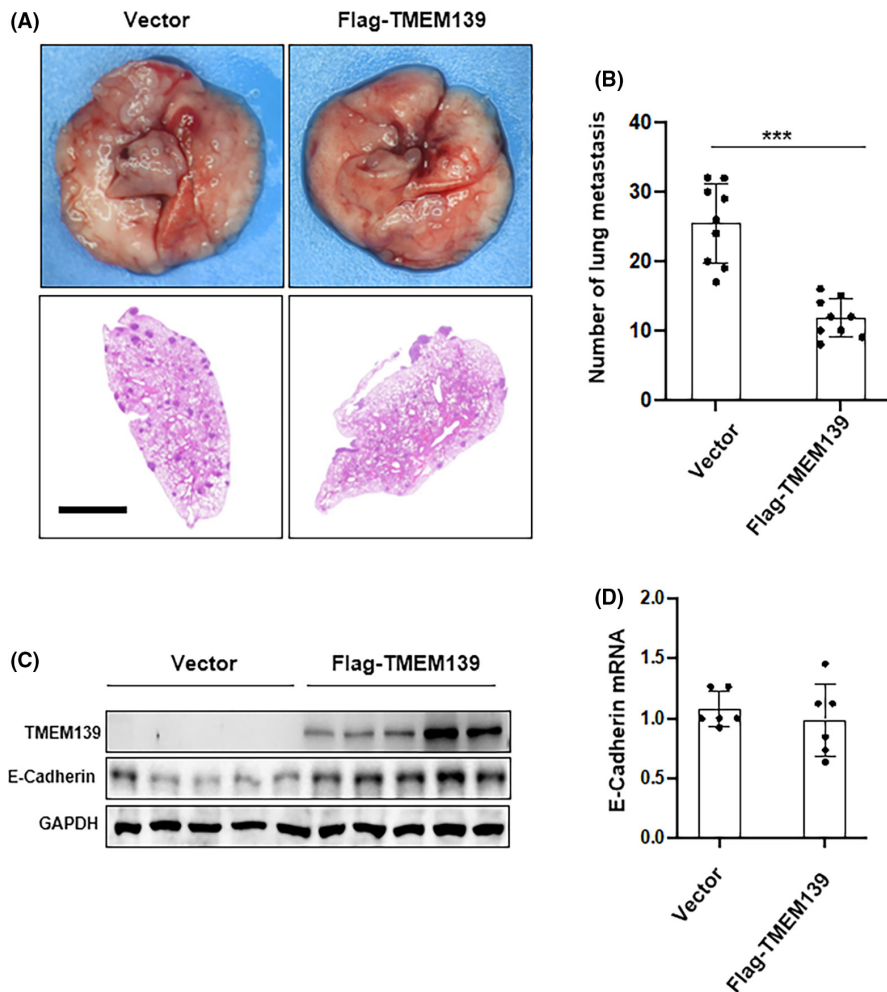


FIGURE 5 TMEM139 inhibits the metastatic potential of lung cancer cells in vivo. (A and B) Nude mouse tumor cell metastasis assay. Photomicrograph of HE-stained lung tissues showing decreased metastatic A549 cell masses in the TMEM139 overexpression group (magnification, 200). (C) Western blotting analysis of TMEM139, E-cadherin, and GAPDH protein expression in xenograft tumor tissue in different groups. (D) qPCR analysis of E-cadherin mRNA expression in xenograft tumor tissue in different groups. *** $P < 0.01$, mean \pm SEM

Numerous TMEMs have been reported to be involved in cancer progression and drug resistance.³¹ Among these TMEMs, some TMEMs, such as TMEM25 and TMEM7, act as tumor suppressors, while others, such as TMEM158 and TMEM14A, act as proto-oncogenes.³²⁻³⁵ In this study, we found that TMEM139 acts as a tumor suppressor in NSCLC and prevents EMT in NSCLC by inhibiting lysosomal degradation of E-cadherin. Whether these TMEMs can interact with or regulate each other needs to be further investigated, which will expand our understanding of cancer development and metastasis, and provide a foundation to develop novel therapeutic strategies.

Our study showed that TMEM139 was significantly downregulated in NSCLC and that reduced expression of TMEM139 was correlated with poor prognosis in NSCLC patients. Mechanistically, TMEM139 directly interacts with E-cadherin and prevents its lysosomal degradation, which inhibits EMT in NSCLC cells both in vitro and in vivo.

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DISCLOSURE

The authors disclose no potential conflicts of interest.

ORCID

Shuai Zhang  <https://orcid.org/0000-0003-2107-719X>

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