ORIGINAL ARTICLE

TMEM107 inhibits EMT and invasion of NSCLC through regulating the Hedgehog pathway

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Abstract
Background: Transmembrane protein 107 (TMEM107) is a key regulator of the cilium composition and Hedgehog signaling. Lower TMEM107 gene copies are correlated with poor prognosis in non-small cell lung carcinoma (NSCLC). However, TMEM107 protein expression, localization, and function in NSCLC remain unclear.

Methods: We first evaluated TMEM107 expression in 12 newly diagnosed cases of NSCLC and paired adjacent healthy tissues by western blotting. We then used an immunohistochemical method to detect TMEM107 expression in 106 paraffin-embedded NSCLC and corresponding normal samples and analyzed its relationship with clinicopathological parameters. Moreover, we determined the impact of TMEM107 upregulation and downregulation on invasion, EMT and Hedgehog pathway in NSCLC cells.

Results: Our results showed that TMEM107 is localized in the cytoplasm and that its expression was lower in NSCLC. TMEM107 expression was positively correlated with cell differentiation and negatively correlated with lymph node metastasis. In A549 and HCC460 cells, downregulation of TMEM107 facilitated cell invasion and upregulated the expression of the Hedgehog pathway target protein Gli1, invasion-associated proteins N-cadherin, vimentin, MMP2, and MMP9, and epithelial-mesenchymal transition (EMT), and inhibited the expression of E-cadherin. Treatment with the Hedgehog pathway inhibitor GANT61 attenuated TMEM107 knockdown-induced EMT and invasiveness.

Conclusions: These results indicate that TMEM107 inhibits EMT and invasion by negatively regulating Hedgehog signaling and that it is downregulated in NSCLC.

Key points:
- TMEM107 expression is lower in NSCLC tissues and correlates with poor prognosis
- TMEM107 inhibits invasion of NSCLC cells
- TMEM107 inhibits EMT of NSCLC cells
- Downregulation of TMEM107 activates the Hedgehog signaling pathway
- Downregulation of TMEM107 promotes EMT and migration in NSCLC by activating the Hedgehog signaling pathway

Introduction
Lung cancer is the most common cancer worldwide. The five-year survival rate for non-small cell lung cancer (NSCLC) is less than 16%.1,2 Even with advancements in basic research, an in-depth understanding of the pathophysiology of lung cancer development is lacking.
The Hedgehog signaling pathway is one of the major regulators of mammalian embryonic development and post-embryonic cell growth and differentiation. It is largely silent in adult vertebrates and is only involved in the maintenance of stem cells and tissue regeneration and repair. However, several studies have shown that the Hedgehog pathway is abnormally activated in a variety of tumors including NSCLC and is closely related to the clinical progress of lung cancer. Further studies showed its activation promotes EMT and elevates invasion and metastasis of cancer cells and plays a vital role in maintaining the stemness of cancer stem cells.

TMEM107 is a relatively newly identified gene which encodes for transmembrane protein 107 (TMEM107), which is localized at the transition zone of the primary cilium and regulates the development and protein content of the cilium. Fibroblasts from patients with mutations in TMEM107 showed a reduced number of cilia, altered cilia length, and impaired Hedgehog signaling in several studies. Christopher et al. and Cela et al. confirmed that TMEM107 deletion could cause significant dysfunction of the Hedgehog signaling pathway in mice, which in turn affects the differentiation and migration of their embryonic cells. Moreover, data from databases indicate that TMEM107 gene expression is lower in lung adenocarcinoma (LUAC) and lung squamous carcinoma (LUSC) than that in normal lung, and lower TMEM107 mRNA expression is associated with poor overall prognosis in the lung cancer population.

To date, the expression and biological functions of TMEM107 protein in human cancers have not been studied. To explore the function of TMEM107 in human NSCLC and its relationship with abnormal activation of the Hedgehog signaling pathway, we studied the expression of TMEM107 in NSCLC tissues by immunohistochemistry and western blotting. We also analyzed the effects of TMEM107 levels on the invasion of lung cancer cell lines and explored the underlying molecular mechanisms.

**Immunohistochemistry**

Tumor specimens were collected and fixed in 10% neutral formalin, embedded in paraffin, and cut into 4 μm thick sections. The slices were heat inactivated in an oven at 70°C for one hour, after which they were quickly deparaffinized in xylene, hydrated with gradient alcohol and treated with citrate buffer (0.01 mol/L) (Maixin-Bio, Shenzhen, China) under high pressure for two minutes. The sections were then immunostained with anti-TMEM107 antibody (1:100 dilution; ab181396) at 37°C for two hours. A biotin-streptavidin HRP detection system (SP-9000, ZSGB-bio, Beijing, China) was used for immuno-detection following the manufacturer’s instructions. Immunostaining was detected using dianminobenzidine (DAB). Finally, the slices were counter-stained using hematoxylin, dehydrated with xylene, and sealed with neutral resin. We used a double-blind method to score TMEM107 staining using the following scoring criteria – 0 (no staining), 1 (weak), 2 (moderate), and 3 (high). Percentage scores were assigned as follows – 0 (0%), 1 (1%–35%), 2 (36%–70%), and 3 (71%–100%). The scores of intensity and percentage of each sample were multiplied to obtain a final score ranging from 0 to 9; tumor samples with a final score ≥6 were considered TMEM107 high expression, and those with scores ≤6 were considered TMEM107 low expression.

**Cell culture**

A549, HCC827, H1975, H1299, H460, and SK-MES-1 were purchased from the Cell Bank of the China Academy of Sciences (Shanghai, China). HBE cells were obtained from ATCC (Manassas, VA, USA). A549, HCC827, H1975, H1299 and H460 cells were cultured in RPMI 1640 medium (BI), SK-MES-1 cells were cultured in OPTI-MEM I (Gibco), and HBE cells were cultured in DMEM High Glucose (BI). All media were supplemented with 10% fetal bovine serum (FBS). The cells were maintained in an incubator with 5% CO2 at 37°C.

**Methods**

**Patient samples**

A total of 106 NSCLC paraffin-embedded tissue samples and 12 pairs of specimens from newly diagnosed lung cancer cases were collected from the Fourth Affiliated Hospital of China Medical University between 2005 and 2015. The patients did not receive any treatment for cancer before surgical resection and sample collection. The study was approved by the Medical Research Ethics Committee of the Fourth Affiliated Hospital of China Medical University.

**TMEM107 modulations and Hedgehog inhibition**

Plasmids pCMV6-AC-GFP and pCMV6-AC-GFP-TMEM107 were purchased from OriGene (Rockville, MD, USA). TMEM107-siRNA and scrambled control siRNA were purchased from GenePharma, Shanghai, China. Hedgehog pathway inhibitor, GANT61 was purchased from Selleck Shanghai, China. Transfection was carried out using Lipofectamine 3000 reagent (Thermo Fisher Scientific) following the manufacturer’s instructions. Cells were treated with 10 μM GANT61 in DMSO 24 hours after
transfection for 24 hours. Control cells were treated with an equivalent volume of DMSO.

**Quantitative real-time PCR**

Quantitative real-time PCR was carried out in a 7500 fast real-time PCR system (Applied Biosystems, Foster City, CA, USA) using SYBR Green PCR master mix in a total volume of 20 μL under the following cycling conditions – 95°C for 30 seconds followed by 40 cycles of 95°C for five seconds and 60°C for 30 seconds. The dissociation step was used to generate a melting curve and confirm amplification specificity. Relative gene expression was calculated using the 2−ΔΔCt method, with GAPDH used as a reference. Primer sequences were as follows – TMEM107 forward, 5′-CCATGTTCAACAGCACCAGA-3′, and reverse 5′-GCCCAAAGACGGTGATCAATA-3′; and GAPDH forward, 5′-GCACCGTCAAGGCTGAGAAC-3′, and reverse 5′-TGTTGAAGACGCCAGTGGA-3′. All experiments were performed in triplicate.

**Western blotting**

Total cellular protein was extracted using the NP-40 lysis buffer (P0013F; Beyotime Biosciences, Shanghai, China) containing a protease-inhibitor PMSF (ST506; Beyotime Biosciences, Shanghai, China) and quantified using an enhanced BCA Protein Assay Kit (P0010; Beyotime Biosciences, Shanghai, China). Proteins (30 μg/lane) were resolved by 10% SDS-PAGE gel, and transferred to 0.45 or 0.22 μm PVDF membranes (Millipore, Billerica, MA, USA). The membranes were blocked in 5% BSA (st023; Beyotime Biosciences, Shanghai, China), and then incubated overnight at 4°C with the appropriate primary antibodies: anti-TMEM107 (1:1000; Abcam; ab181396), E-cadherin (1:500; cell signaling technology; 3195), N-cadherin (1:500; Cell Signaling Technology; 13 116), vimentin (1:500; Cell Signaling Technology; 5741), MMP2 (1:500; Cell Signaling Technology; 87809), MMP9 (1:500; Cell Signaling Technology; 13667), and GAPDH (1:1000; Beyotime; AF0006). Membranes were washed with Tris-buffered saline with Tween-20 and then incubated with horseradish peroxidase-conjugated anti-mouse/rabbit IgG (1:2000; ZSGB-BIO, Beijing, China) at room temperature for one hour. Target proteins were detected by ECL (Thermo Fisher Scientific, Waltham, MA, USA) using a BioImaging system (UVP, Inc., Upland, CA, USA).

**Matrigel invasion assay**

Cell invasion assay was performed in 24-well transwell chambers with an 8 μm pore membrane insert (Costar, Washington, DC, USA). The inserts were coated with 50 μL Matrigel (1:7 dilution; BD Biosciences, San Jose, CA, USA). After transfection for 24 hours, the cells were trypsinized, and 5 × 10⁴ cells in 200 μL medium containing 2% FBS were transferred to the upper chamber for 48 hours, and 600 μL medium containing 10% FBS was added to the lower chamber. Cells that penetrated the filter were fixed with 4% paraformaldehyde and stained with hematoxylin. The membrane inserts were observed under the microscope, and the number of cells in 10 randomly selected fields were counted.

**Oncomine analysis**

Oncomine datasets (https://www.oncomine.org/resource/login.html) is an online cancer microarray database. We used it to analyze the gene copies and mRNA expression of TMEM107 in lung cancer specimens and their normal controls. The cutoff threshold of P-value and fold changes were defined as 0.05 and two-fold, respectively.

**Statistical analysis**

SPSS 13.0 software (SPSS Inc., Chicago, IL, USA) was used for all statistical analysis. Kaplan-Meier plotter database21 (http://kmplot.com/analysis/) and the SurvExpress platform22 (http://bioinformatica.mty.itesm.mx/SurvExpress) were used to obtain survival of NSCLC patients in relation to TMEM107 expression level. Chi-square test was used to analyze the correlation between the immunohistochemical expression of TMEM107 and the clinicopathological parameters. Student’s t test was used for assessing the difference between groups, and P < 0.05 was considered statistically significant.

**Results**

**TMEM107 expression is lower in NSCLC tissues and correlates with poor prognosis**

We first analyzed TMEM107 expression in human cancers and their normal controls according to data in the Oncomine. Results showed that the gene copies of TMEM107 were significantly downregulated in many kinds of cancers, including in LUAC and LUSC (Fig 1a,b), and that the mRNA expression of TMEM107 was negatively correlated with tumor grade and N stage in lung cancer (Fig 1c). Next, we analyzed the protein expression of TMEM107 by western blotting and immunohistochemistry. Results verified that TMEM107 protein expression was indeed downregulated in NSCLC (Fig 2a–d). The expression of TMEM107 was positively correlated with tumor differentiation (P = 0.010) and negatively correlated with lymph node metastasis (P = 0.032), but not significantly
Figure 1 Expression of TMEM107 in lung cancer analyzed by Oncomine datasets. (a) The gene copies of TMEM107 were significantly downregulated in many kinds of cancers. (b, c) The gene copies of TMEM107 were downregulated in LUAC and LUSC in Weiss and TCGA lung statistics. (d) The mRNA expression of TMEM107 was negatively correlated with tumor grade and N stage in lung cancer.
correlated with age ($P = 0.732$), gender ($P = 0.965$), histological type ($P = 0.84$), T stage ($P = 0.825$), or clinical stage ($P = 0.274$) (Table 1). Last, we used the Kaplan-Meier plotter database and the survExpress database to evaluate the prognostic value of TMEM107 in patients with lung cancer. Kaplan-Meier plotter test showed that the overall survival of patients with lower TMEM107 mRNA level was shorter compared to those with higher TMEM107 mRNA level (Fig 2e). SurvExpress analysis showed that the expression of TMEM107 mRNA in the high risk group was
significantly lower than in the low risk group (Fig 2f,g). “High” and “low” risk groups were divided on the basis of the maximized risk algorithm. These results indicate that TMEM107 can be an independent prognostic predictor in NSCLC.

**TMEM107 inhibits invasion of NSCLC cells**

Immunohistochemical analyses confirmed that TMEM107 expression was negatively correlated with lymph node metastasis. In order to verify the impact of TMEM107 on the invasive potential of lung cancer, we upregulated or knocked down TMEM107 expression in lung cancer cells and evaluated their invasiveness (Fig 3). First, we used the immortalized lung bronchial epithelial cell line HBE to detect the baseline expression of TMEM107. Next, TMEM107 expression in six NSCLC cell lines—HCC827, H460, H1975, SK-MES-1, H1299, and A549—were compared against TMEM107 expression in HBE cells (Fig 3a,b). All NSCLC cell lines except HCC827 showed a lower expression of TMEM107 than that in HBE cells, which is consistent with our TMEM107 expression in lung cancer tissue. HCC827 and H1975 are NSCLC cell lines with epidermal growth factor receptor (EGFR) activated mutations, in which the activated EGF receptors initiate the activation of various signaling pathways. The abnormal over-activated signaling may make the results of subsequent experiments on cellular function uncertain. Therefore, the HCC827 and H1975 cell lines were not selected for the present study. We selected A549 and H460 with relatively high and low TMEM107 expression, respectively, for subsequent experiments.

TMEM107 expression was upregulated using the pCMV6-AC-GFP vector (Fig 3c,e,g) or downregulated using a siRNA (Fig 3d,f,h) in A549 and H460 and the effect on invasiveness was monitored using a Matrigel invasion assay. Knockdown of TMEM107 promoted the invasion of A549 and H460, while TMEM107 overexpression significantly reduced the invasiveness (Fig 3i). Consistent with these results, TMEM107 upregulation downregulated the levels of matrix metalloproteinases (MMP2 and MMP9) which are essential proteins that contribute to the invasiveness of cancer cells. In contrast, the downregulation of TMEM107 expression increased MMP2 and MMP9 levels. These results indicate that TMEM107 can inhibit invasion of NSCLC cells.

**TMEM107 inhibits EMT of NSCLC cells**

Immunohistochemical analyses and functional studies suggested that TMEM107 expression is related to differentiation and invasion of NSCLC. Since EMT is a crucial mechanism involved in dedifferentiation, invasion, and metastasis of cancer cells, we investigated the morphological changes of A549 and H460 cells and the changes in expression of EMT-related proteins after upregulating and downregulating TMEM107. Our results show that TMEM107 expression did not significantly affect the morphology of lung cancer cells. On the other hand, TMEM107 knockdown increased the expression of N-cadherin and vimentin and decreased the expression of E-cadherin which are EMT-related proteins. Results were reversed with TMEM107 upregulation (Fig 3m,n). These results indicate that TMEM107 can inhibit EMT of NSCLC cells.

**Downregulation of TMEM107 activates the Hedgehog signaling pathway**

Studies have shown that the activation of the Hedgehog signaling pathway can promote EMT in tumor cells. It has also been reported that TMEM107 deletion could activate Hedgehog signaling pathway in human and mice. Our results demonstrated that TMEM107 knockdown induced EMT in lung cancer cells. Therefore, we speculated that the effect of TMEM107 on invasion and EMT in A549 and H460 cells might be mediated through the Hedgehog signaling pathway. As Gli1 is a highly reliable tool for verifying Hedgehog signaling pathway activation, we used Gli1 as a marker to verify the effect of TMEM107 on the Hedgehog signaling pathway.
Figure 3 TMEM107 inhibits invasion and EMT of NSCLC cells. (a) TMEM107 and Gli1 levels in HBE cells and six NSCLC cell lines determined by western blot analysis. (b) Densitometric quantification of western blot results. (c) Western blot analysis of transfection efficiency and (d) knockdown efficiency of TMEM107 in A549 and H460 cells. (e) Densitometric quantification of western blot results from (c) and (d), respectively. (g, h) q-RT-PCR assay for transfection efficiency. (i) Transwell assay to assess cell invasion after TMEM107 knockdown and overexpression. (j) The histogram of the number of cells invaded. (k) Expression of invasion-related proteins after TMEM107 downregulation and overexpression. (l) Densitometric quantification of western blot results from (k). (m) Expression of EMT-related proteins after TMEM107 upregulation and downregulation. (n) Densitometric quantification of western blot results from (m) (*P < 0.05). (a) TMEM107, (b) Gli1; (c) p-NC, (d) p-107; (e) Si-NC, (f) Si-107; (g) p-NC, (h) p-107; (i) Si-NC, (j) Si-107; (k) Si-NC, (l) si-TMEM107; (m) p-NC, (n) p-TMEM107.
indicator of Hedgehog signaling activation and can positively regulate it through a positive feedback loop mechanism,\textsuperscript{26} we not only detected the expression of Gli1 in HBE and six NSCLC cell lines (HCC827, H460, H1975, SK-MES-1, H1299, and A549) (Fig 3a,b) to observe how is the expression correlation of TMEM107 and Gli1 of lung...
cancer cells, but also monitored the change in expression of Gli1 after upregulating or downregulating TMEM107. In the above cell lines, although Gli1 appeared to be negatively correlated with TMEM107 expression ($R = -0.501$), no statistically significant association was observed between them ($P = 0.252$). However, an increase in Gli1 expression was observed when TMEM107 was knocked down, which was reversed when TMEM107 was upregulated (Fig 4a,b). We therefore determined that TMEM107 could negatively influence the Hedgehog signaling pathway.

**Downregulation of TMEM107 promotes EMT and migration in NSCLC by activating the Hedgehog signaling pathway**

Next, we determined whether inhibiting Hedgehog signaling would affect EMT and invasiveness in TMEM107 knockdown cells. We first screened the A549 cell line and confirmed that the optimal inhibitory concentration of the Hedgehog signaling pathway inhibitor GANT61$^{8,27}$ was 10 μM (Fig 4c,d). As shown in Fig 4e and f, GANT61 treatment attenuated the TMEM107-knockdown-induced invasiveness of A549 and H460 cells. Consistent with this, we observed that GANT61 treatment inhibited the expression of Gli1, MMP2, MMP9, N-cadherin, and vimentin and increased E-cadherin expression in TMEM107-knockdown NSCLC cells (Fig 4g,h). To verify the above results, we performed Hedgehog inhibition studies in cells with upregulated TMEM107 and observed that the invasiveness and the expression of EMT-related proteins were not significantly affected by GANT61 treatment (results not shown). All the above experiments were rigorously performed three times and the results could be replicated. Finally we were able to conclude that TMEM107 inhibits EMT and invasion by suppressing Hedgehog signaling and that it is downregulated in NSCLC.

**Discussion**

In humans, data from the Oncomine database indicate that the expression of **TMEM107** gene is lower in LUAC and LUSC than that in normal lung and that the **TMEM107** mRNA is lower in hemangioblastoma, anaplastic large cell lymphoma, testicular seminoma, and embryonal carcinoma than that in healthy lymphoid tissue and testicular tissue. However, the protein expression of TMEM107 in tumors remains unclear. In this study, using immunohistochemistry and western blot analyses, we confirmed that TMEM107 expression is lower in human lung cancer. Clinicopathological correlation analyses revealed that TMEM107 expression was positively correlated with differentiation and negatively correlated with lymph node metastasis of NSCLC. Our results, together with the data from databases, suggest that TMEM107 is a tumor suppressor gene. The poor prognosis of lung cancer patients with lower TMEM107 expression learned from Kaplan-Meier and SurvExpress database supports our hypothesis.

MMP-2/9, known as gelatinase A/B, can degrade extracellular matrix (ECM) proteins such as the basement membrane and the surrounding stroma to make tumor cells take the first step of invasion.$^{25}$ In our experiment, downregulation of TMEM107 expression increased MMP2/9 levels and promoted invasion of lung cancer cells. This is consistent with the results of Christopher et al. and Cela et al. where **TMEM107** deletion inhibited differentiation and promoted migration of neuronal stem cells in mouse embryos.$^{15,16}$

The invasiveness of **NSCLC** has been shown to be related to the acquisition of EMT.$^{9}$ We found that TMEM107 knockdown made A549 and H460 cells lose E-cadherin and gain N-cadherin and vimentin which are important symbols of occurrence of EMT,$^{28}$ suggesting a novel mechanism underlying the involvement of TMEM107 in invasion of NSCLC.

The Hedgehog pathway can be induced through a canonical and noncanonical mode. They both activate downstream GLI transcription factors (Gli1, Gli2, Gli3) which migrate to the nucleus to activate Hedgehog target genes. Unlike Gli2 and Gli3, Gli1 is not only a transcription factor but also a direct transcriptional Hedgehog target gene. Induction of Gli1 expression is a reliable marker for Hedgehog pathway activity.$^{7,8,26}$ Cela et al. reported that Gli1 gene copies were increased in **TMEM107**−/− mice,$^{16}$ Shaheen et al. showed that Gli1 mRNA were induced in the fibroblasts of a ciliopathy patient with the TMEM107 loss-of-function mutation.$^{39}$ Since the Hedgehog signaling pathway is notably involved during embryonic development, tissue repair, tumorigenesis and other processes, and can be induced by canonical and noncanonical modes, the expression state of Gli1 in cells must be very complex and variable. Therefore, we did not find a significant correlation ($P = 0.252, R = -0.501$) between Gli1 and TMEM107 expression in seven unregulated lung cell lines. Fortunately, our other results confirmed that downregulation of **TMEM107** increased Gli1 protein in NSCLC cell lines, indicating **TMEM107** knockdown activates the Hedgehog signaling pathway, which is consistent with previous results.

Various studies have shown that the Hedgehog pathway is activated in NSCLC$^{7}$ and the activated Hedgehog pathway has been reported to be involved in the functions that induce EMT and promote invasion.$^{7}$ Inhibition of Hedgehog signaling reduced the viability of NSCLC cells.$^{29,30}$ Further analyses showed that treatment with the Hedgehog pathway in NSCLC inhibitors ameliorated TMEM107
downregulation-stimulated EMT suggesting that TMEM107 is involved in the regulation of EMT of lung cancer cells through the Hedgehog signaling pathway.

On account that previous studies have reported that TMEM107 mutants decreased numbers of cilia and upregulated Hedgehog signaling in human and mice cells,15–19 we suspect that TMEM107 knockdown may activate Hedgehog signaling by decreasing the number of primary cilia in lung cancer cells. Although somewhat counterintuitive, this seemingly paradoxical effect is consistent with the alternative activating or inhibitory role of cilia in mediating the Hedgehog signaling pathway.31,32 But theory does not mean reality, and how TMEM107 downregulation activates the Hedgehog signaling pathway will be addressed in our future studies.

Together, our research demonstrated that TMEM107 plays an important role in promoting cancer development and is a potential prognostic marker for NSCLC. Therefore, targeting TMEM107 might be a potential therapeutic strategy for controlling NSCLC.

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Disclosure

The authors declare that they have no conflict of interest.

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