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*Original Article*

# siRNA-TMEM98 inhibits the invasion and migration of lung cancer cells

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**Abstract:** Non-small cell lung cancer (NSCLC) is one of the most common human malignancies, which threatens people's life heavily. Recently, TMEM98 is supposed to be of great value for the discoveries of anti-tumor drugs. We attempt to explore the biological role of TMEM98 in the human lung carcinoma. Clinical lung cancer tissue and normal tissue were collected, and the mRNA expression of TMEM98 in cancer tissue was significantly higher than that in normal tissue. By real-time-PCR and Western blot analysis of TMEM98 expression, human A549 and H460 cells were determined to carry out further investigations. By CCK8, it is found that siRNA-TMEM98 treatment effectively suppressed the proliferation of A549 and H460 cells. In addition, the invasion and migration of A549 and H460 cells were also inhibited by siRNA-TMEM98. We then studied the invasion and migration related proteins level by Western blot. From our result, the protein expression of MMP-2, MMP-9, RhoC and MTA1 were all regulated dramatically in siRNA-TMEM98 groups compared with the control and mock group. To conclude, our results indicated that siRNA-TMEM98 inhibited the invasion and migration of lung cancer cells, which can be considered as a novel target for NSCLC treatment.

**Keywords:** TMEM98, invasion, migration, lung cancer

## Introduction

Non-small cell lung cancer (NSCLC) is one of the most common human malignancies threatening people's life and its incidence continues to rise. The most common cause of lung cancer is smoking and nearly 87% of lung cancer cases are caused by smoking. The NSCLC metastasis is reported as the most important cause leading to the failure of treatment [1, 2]. Chemotherapy, radiotherapy and surgical treatment are widely used for NSCLC therapy. However, the cure rate of NSCLC is very low because of the postoperative complication, side effects and recurrence.

There is little report about human TMEM98 gene. Its chromosome localization is in 17q11.2. There are two RNA splicing forms released in the NCBI database, TMEM98-v1 and TMEM98-v2. Although there is slightly difference between them in the 5' untranslated region, the coding products of them are almost the same, which consists of 226 amino acid and the molecular weight is 24.6 kDa, isoelectric point is 4.718. There is a high homologous among different species. Comparing with the amino acid sequence of the mouse, the homology is as high as 98.7%.

It is predicted that TMEM98 is type I membrane molecular by TMHMM analysis. By Signal P analysis, it found that there was typical signal peptide consist of 21 amino acid in its N-terminal, which implied that TMEM98 might probably be a new secretory protein [3]. It appears up regulated under the stimulation of inflammation. This suggests that there should be some relationships between TMEM98 and the development of inflammation. There is a closed relationship between inflammation and tumor. Many tumor progressions are accompanied with inflammatory injuries; and inflammatory microenvironment can promote the survival and the progress of tumor cells. Therefore, TMEM98 will be of great value for the discoveries of the new target of the treatments to inflammations and anti-tumor drugs.

**Table 1.** Primers used in qRT-PCR analysis

| Gene | Primer sequence | Species |
|---|---|---|
| TMEM98 | Forward: 5'-CAATTGAGCTTCCACCTG-3' | Human |
| | Reverse: 5'-CCGACAGAGCTCTAGAGAAC-3' | |
| GAPDH | Forward: 5'-CACCCACTCCTCCACCTTTG-3' | Human |
| | Reverse: 5'-CCACCACCCTGTTGCTGTAG-3' | |

In the present study, we attempt to explore the effect of siRNA-TMEM98 silencing on migration and invasion in human lung cancer cells which expressed TMEM98 notably. Moreover, the mechanisms involved are also investigated to provide new thoughts for the treatment of NSCLC.

## Materials and methods

*Patients and tissue samples*

38 lung cancer patients admitted to Zhongnan Hospital were enrolled in this study. All patients have complete clinical and pathological follow-up data. Adjacent normal lung tissues were also collected as negative controls. These normal lung tissues were resected within at least 2 cm of the tumor margin when the patients underwent definitive surgery. Ethical approval for the study was provided by the independent ethics committee, Zhongnan Hospital. Informed and written consent was obtained from all patients or their advisers according to the ethics committee guidelines.

*Cell culture*

H1229, H446, A549, SPCA-1 and H460 cells are human lung carcinoma cells. All cells were obtained from the Shanghai Cell Bank, Chinese Academy of Sciences (Shanghai, China). Cells were cultured in DMEM supplemented with 10% fetal bovine serum, 100 units/mL penicillin and 100 μg/mL streptomycin, and incubated in a humidified atmosphere at 37°C with 5% $CO_2$.

*miRNA transfection*

Cells were seeded in antibiotic-free medium the day before transfection. The cells were transfected with 50 nmol/L of TMEM98-siRNA or negative control by using lipofectamine™ 2000 (Invitrogen, Shanghai, China) according to the instructions provided by the manufacturer. After 48 hours, the transfected cells were collected and processed for quantitative real-time PCR (qPCR), western blot, proliferation, cell cycle, migration and invasion assay.

*Cell proliferation assay*

Cell viability was assessed by Cell Counting Kit (CCK)-8 kit (Tongren, Shanghai, China). Briefly, $4 \times 10^3$ cells were seeded in each 96-well plate, and further incubated for 24 and 48 hours, respectively. CCK-8 reagent was added to each well at 1 hour before the endpoint of incubation. The optical density (OD) 450 nm values in each well were determined by a microplate reader. Experiments were repeated at least three times each time in triplicate.

*Cell invasion assay*

Cell invasion assay was performed by a 24-well Transwell chamber with a pore size of 8 μm (Sigma, San Francisco, USA). The inserts were coated with 50 μL Matrigel (dilution at 1:2; BD Bioscience). Cells were trypsinised after transfection for 24 hours and transferred to the upper Matrigel chamber in 100 μL of serum-free medium supplementing $1 \times 10^5$ cells and incubated for 24 hours. The lower chamber was filled with medium containing 10% FBS as chemo attractants. After incubation, the cells that passed through the filter were fixed and stained by 0.1% crystal violet. The numbers of invaded cells were counted in five randomly selected high power fields under a microscope (Olympus, Shenzhen, China).

*Migration assay*

Cells in logarithmic phase were digested by 0.25% trypsin (Gibco, Shanghai, China) and then suspended in RPMI-1640 (Hyclone) medium containing 10% fetal calf serum (Gibco). Cells were seeded in a 12-plate microplate at a density of $1 \times 10^5$ cells/ml and then incubated for 1 h. The supernatant was discarded and cells were washed 2 times by PBS (Gibco). 4% paraformaldehyde (Thermo, Waltham, USA) was supplemented for 15 min and cells were stained by Giemsa (Thermo, Waltham, USA) for 30 min. Then cells were washed several times and the optical density (OD) values were read at 570 nm by a microplate reader (Thermo, Waltham, USA). Adhesion rate (%) = $(OD_1/OD_0) \times 100\%$, $OD_1$: HB treated groups; $OD_0$: control group.

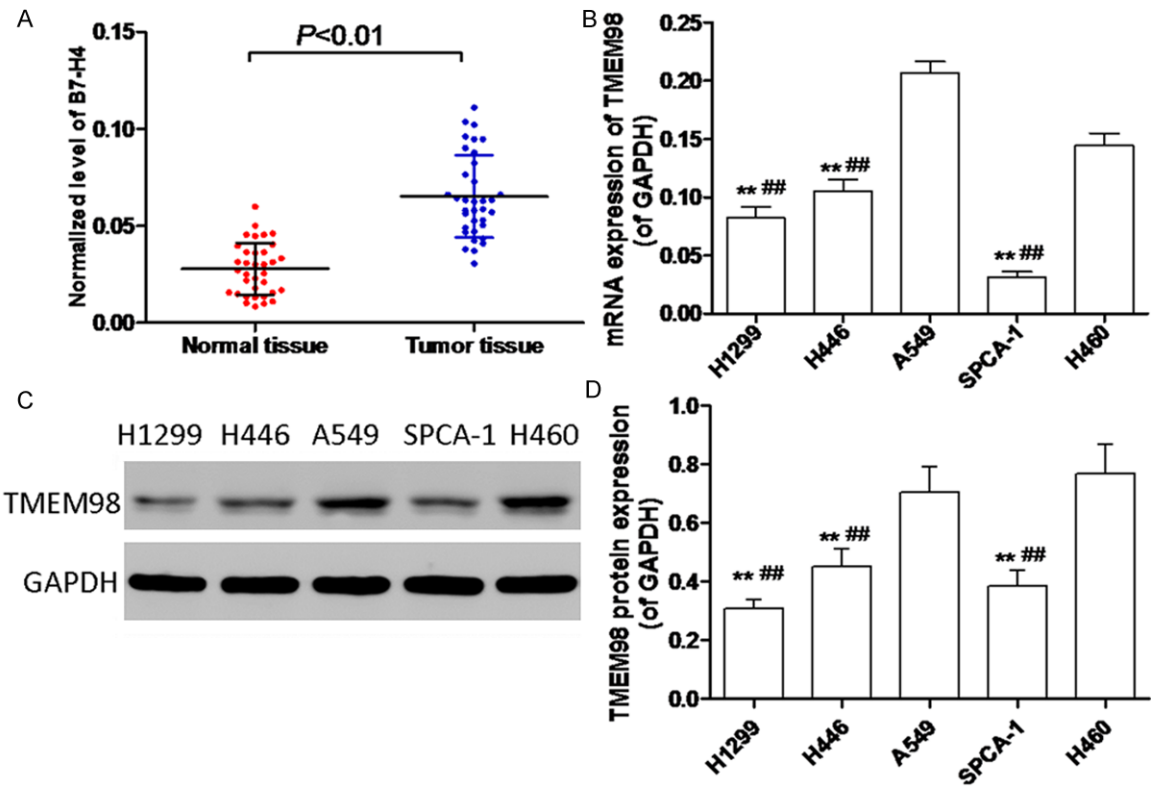


**Figure 1.** Expression of TMEM98 in human lung cancer tissue and cells. A. 37 lung cancer tissues and their adjacent normal tissues were collected and mRNA expression of TMEM98 mRNA was identified by RT-PCR. B. mRNA expression of TMEM98 mRNA in H1229, H446, A549, SPCA-1 and H460 cells was detected by RT-PCR. C and D. Protein level of TMEM98 mRNA in H1229, H446, A549, SPCA-1 and H460 cells was assessed by Western blot. $^{**}P < 0.01$ compared with A549 cell line; $^{\#\#}p < 0.01$ compared with H460 cell line; data are expressed as the mean ± SD, n = 6.

*Reverse transcription and real-time PCR*

The mRNA expression of TMEM98 in cells after TMEM98-siRNA transfection was quantified by RT-PCR. Total RNA was isolated from cells using Trizol Reagent (Invitrogen) and quantified. cDNA was synthesized from 5 mg of RNA using AMV reverse transcriptase (Fermentas, USA) according to the manufacturer's instructions. TMEM98 was amplified from the cDNA by RT-PCR. The PCR conditions consisted of 5 min at 95°C one cycle, 30 seconds at 95°C, 30 seconds at 55°C, 30 seconds at 72°C, and 7 min at 72°C 35 cycles. Primer pairs for human genes were designed using the Primer Express Software (Applied Biosystems, Shanghai, China) and are listed in **Table 1**.

*Western blot*

Cultured or transfected cells were harvest and washed twice with PBS and lysed in ice-cold radio immunoprecipitation assay buffer (RIPA, Beyotime, Shanghai, China) with freshly added 0.01% protease inhibitor cocktail (Sigma, Shanghai, China) and incubated on ice for 30 min. Cell lysis was centrifuged at 13,000 rcf for 10 min at 4°C and the supernatant (20-30 μg of protein) was run on 10% SDS-PAGE gel and transferred electrophoretically to a polyvinylidene fluoride membrane (Millipore, Shanghai, China). The blots were blocked with 5% skim milk, followed by incubation with antibodies against TMEM98 (Abcam), GAPDH (Fermentas), MMP-2 (Abcam), MMP-9 (Abcam), RhoC and MTA1 (CST). Blots were then incubated with goat anti-mouse or anti-rabbit secondary antibody (Beyotime, Shanghai, China) and visualized using enhanced chemiluminescence (ECL, Thermo Scientific, Shanghai, China).

*Statistic analysis*

All results are presented as the mean ± SD and the data were analyzed by a SPSS 13.0 statistical package (SPSS Inc., Chicago, IL). Data for

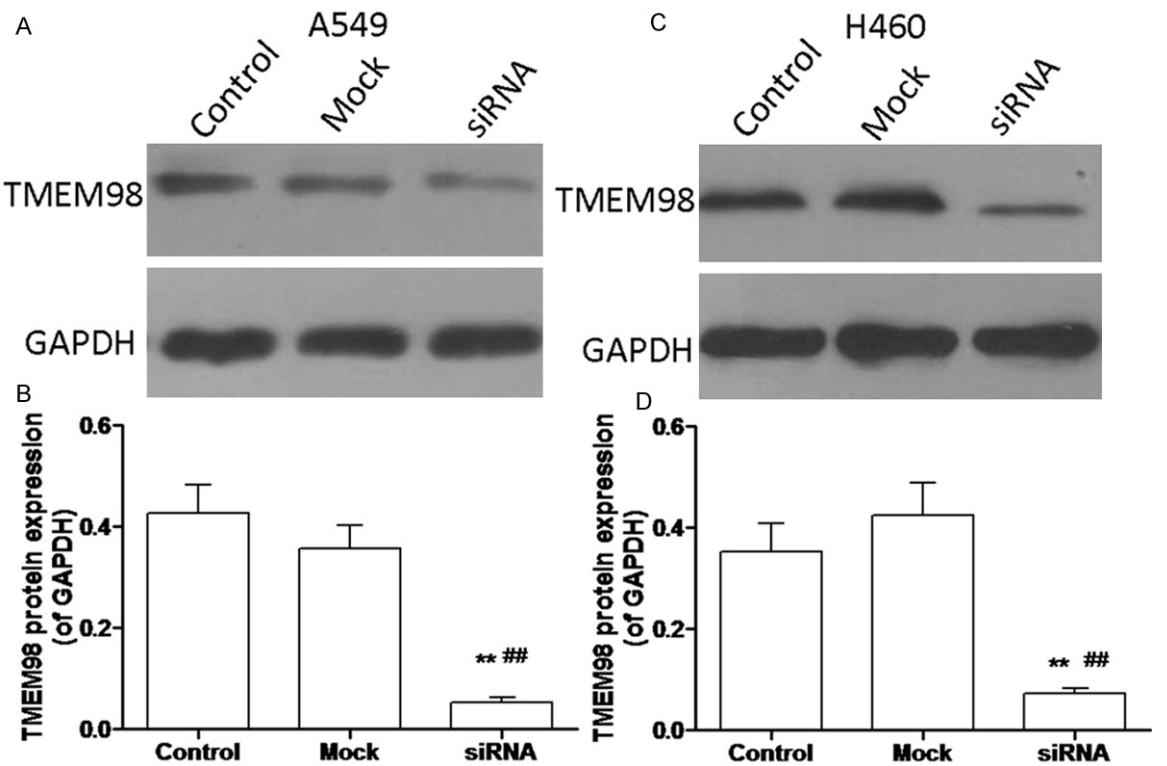


**Figure 2.** Protein expression of TMEM98 in A549 and H460 cells. A and B. After TMEM98-siRNA transfection for 48 h, Protein expression of TMEM98 in A549 cells was quantified by Western blot analysis. C and D. After TMEM98-siRNA transfection for 48 h, Protein expression of TMEM98 in H460 cells was quantified by western blot analysis. $^{**}P < 0.01$ compared with the control cells; $^{\#\#}P < 0.01$ compared with the mock cells; data are expressed as the mean ± SD, n = 6.

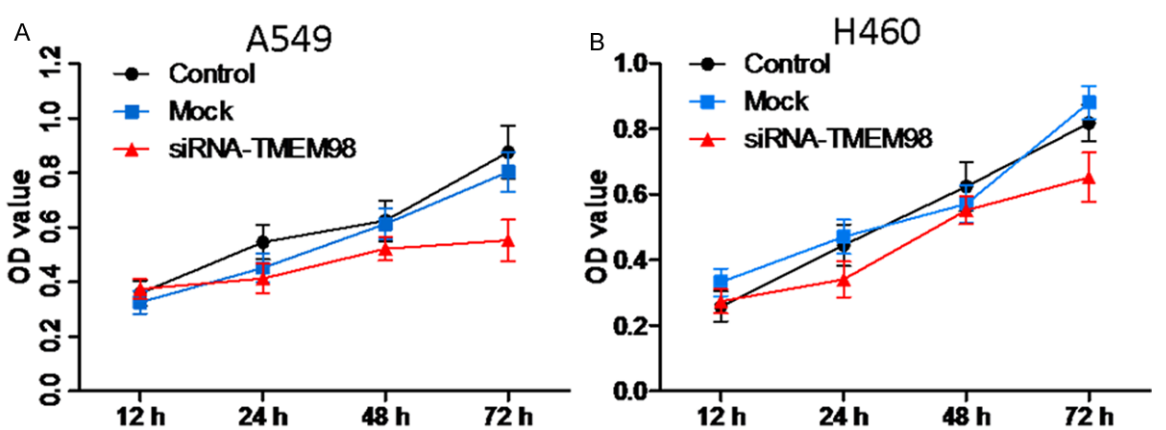


**Figure 3.** Effect of siRNA-TMEM98 on the proliferation of A549 and H460 cells. A and B. After TMEM98-siRNA transfection for 12, 24 48 and 72 h, cell viability of A549 and H460 cells was identified by flow cytometry. $^{**}P < 0.01$ compared with the control cells; $^{\#\#}P < 0.01$ compared with the mock cells; data are expressed as the mean ± SD, n = 6.

multiple comparisons were subjected to one-way ANOVA followed by Dunnett's test and a value of $P < 0.05$ was considered statistically significant.

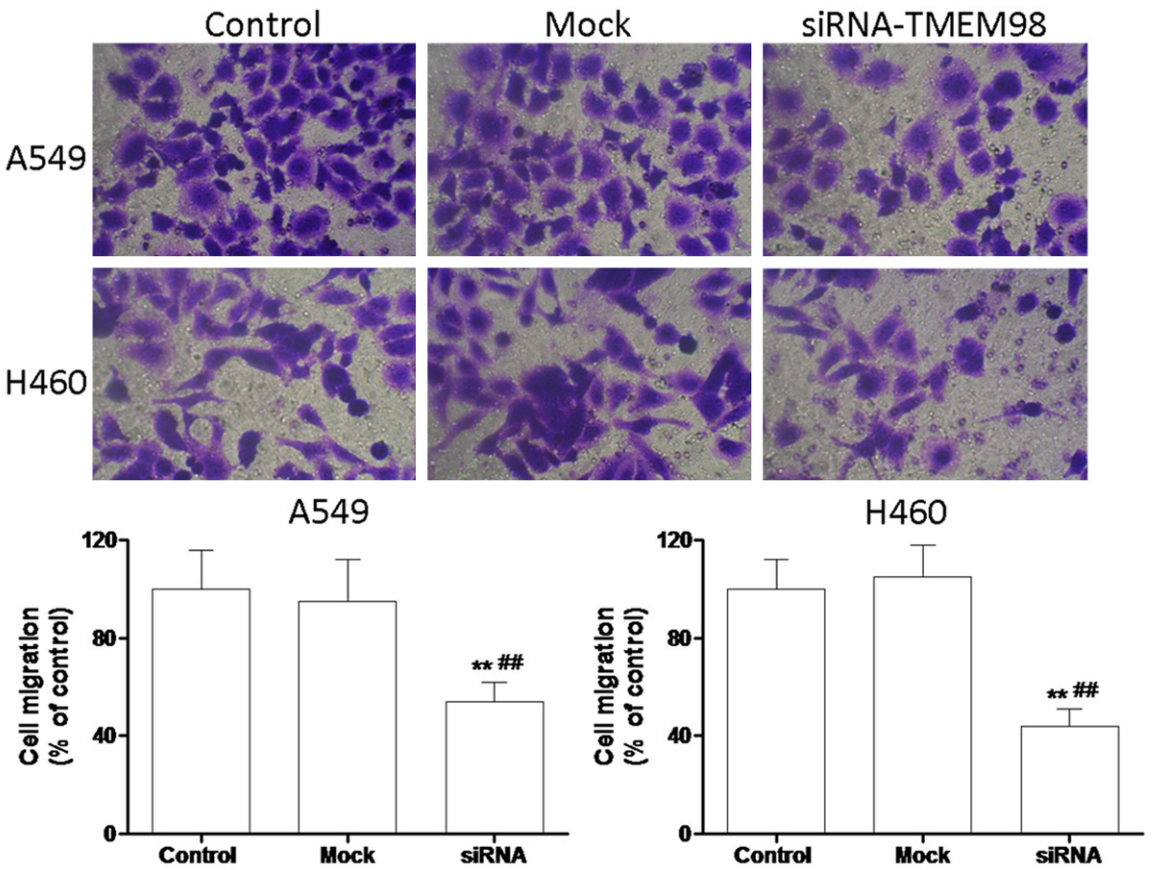


**Figure 4.** Effect of siRNA-TMEM98 on the invasion of A549 and H460 cells. After TMEM98-siRNA transfection for 48 h, invasive ability of human A549 and H460 cells was identified by transwell assay. $^{**}P < 0.01$ compared with the control cells; $^{\#\#}P < 0.01$ compared with the mock cells; data are expressed as the mean ± SD, n = 6.

## Results

### *Expression of TMEM98 in lung cancer tissues and normal tissues*

To verify the biological role of TMEM98 in lung carcinoma, we used real-time PCR to detect the expression levels of TMEM98 in lung cancer patients' tissues. We collected 35 lung carcinoma tissues and their adjacent normal tissues. As **Figure 1A** shows, TMEM98 expression level was higher in lung tumor tissues than that of adjacent normal tissue control ($P < 0.01$).

### *TMEM98 expression in human lung cancer cells*

An obvious difference was presented in lung cancer tissue and normal tissue. We then detected the mRNA expression and protein level of TMEM98 in various lung carcinoma cell lines including H1229, H446, A549, SPCA-1 and H460 cells by RT-PCR and western blot, respectively. As shown in **Figure 1B**, TMEM98 mRNA expressions in A549 and H460 cell lines were significantly higher than any other cell line. In addition, western blot displayed that protein level of TMEM98 was the highest among all the cell line (**Figure 1C** and **1D**). As a result, A549 and H460 cell lines were determined to carry out further investigations.

### *siRNA-TMEM98 inhibited the proliferation of A549 and H460 cells*

TMEM98 mRNA was interfered in A549 and H460 cells as previously described. Western blot was employed to identify the interference efficient. Western blot showed that TMEM98 protein level was declined notably in TMEM98 siRNA group in comparison with the control group and mock groups in both A549 and H460 cells (**Figure 2**).

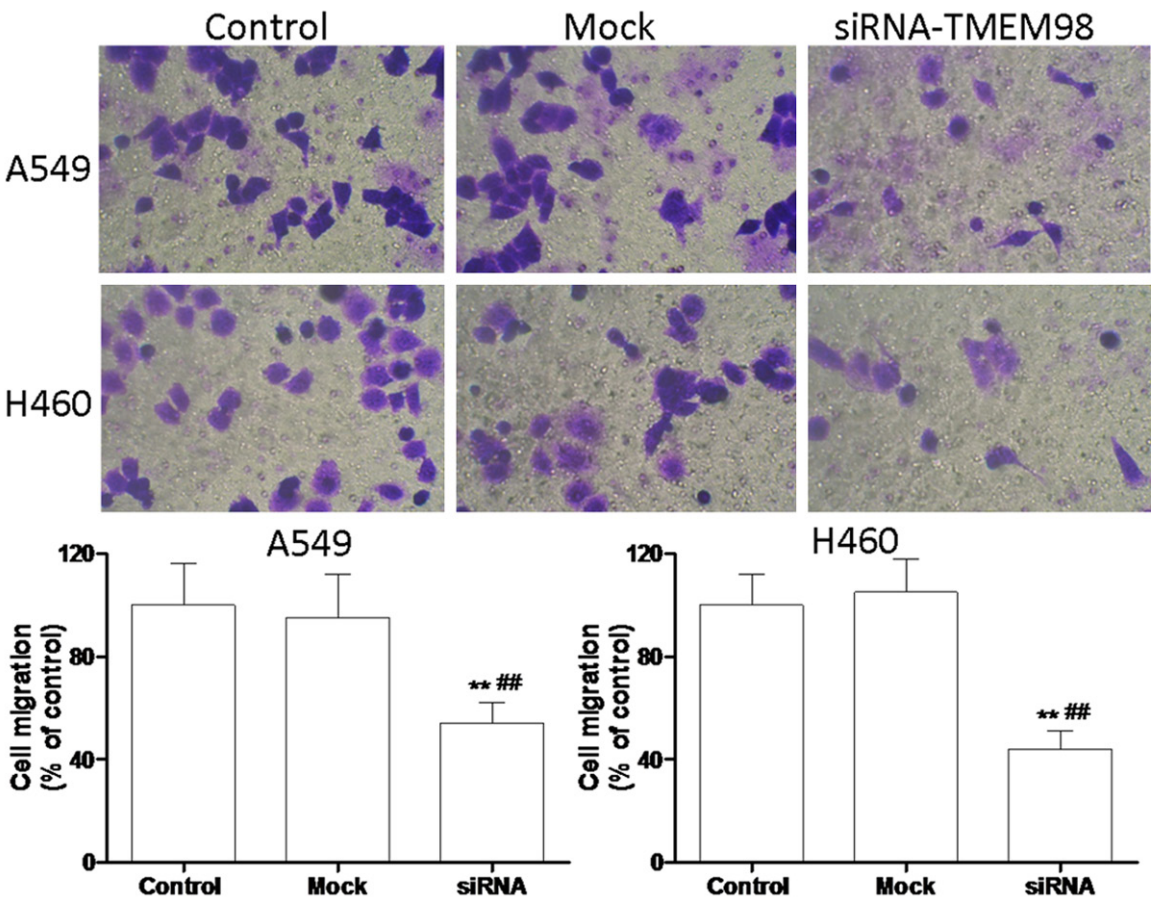


**Figure 5.** siRNA-TMEM98 depressed the migration of A549 and H460 cells. A549 and H460 cells were tranfected after 48 h, cell migration was detected as previously described. $^{**}P < 0.01$ compared with the control cells; $^{\#\#}P < 0.01$ compared with the mock cells; data are expressed as the mean ± SD, n = 6.

In addition to explore the effect of siRNA-TMEM98 on the proliferation of human A549 and H460 cells, CCK8 assay was employed to identify the cell proliferation. As shown in **Figure 3A**, proliferation of siRNA-TMEM98 human A549 cell group was significantly depressed compared with control and mock groups at 72 h. In **Figure 3B**, siRNA-TMEM98 also markedly suppressed the proliferation of human H460 cells compared with control and mock groups after transfection for 72 hours.

*siRNA-TMEM98 suppressed the migration and invasion of A549 and H460 cells*

Cell migration and invasion were also studied by transwell assay. It is showed in **Figure 4**, the invasive ability of siRNA-TMEM98 group was degraded notably in comparison with the control and mock group of A549 and H460 cells. In **Figure 5**, the migration rate of siRNA-TMEM98 group was obviously lower than that of control and mock group of A549 and H460 cells.

*Expression of MMP-2, MMP-9, RhoC and MTA1 was regulated by siRNA-TMEM98*

MMP-2, MMP-9, RhoC and MTA1 are identified as invasion and migration related genes. Therefore, we further detect the protein expression by Western blot. As for human A549 cells, MMP-2, MMP-9, RhoC and MTA1 expression were all suppressed by siRNA-TMEM98 compared with the control and mock groups (**Figure 6A** and **6B**). It is shown in **Figure 6C** and **6D**, compared with the control and mock groups, MMP-2, MMP-9, RhoC and MTA1 expression in H460 cell were also attenuated markedly by siRNA-TMEM98 treatment.

## Discussion

In 2008, Chinese National Human Genome Center analyzed the Genome Database in order to obtain novel cytokines. They selected many candidates and verified many kinds of proteins. Human TMEM98 (transmembrane protein 98)

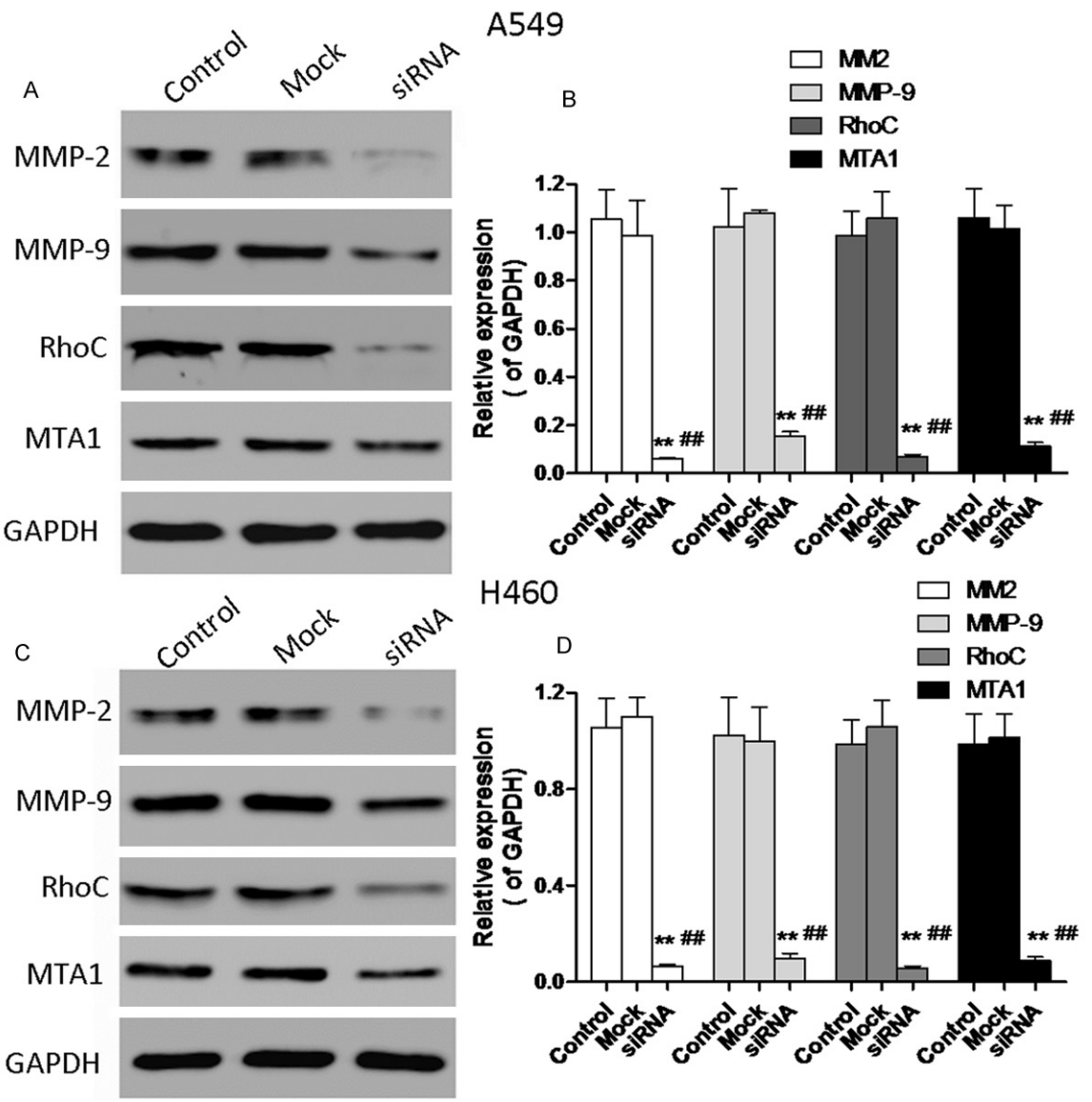


**Figure 6.** siRNA-TMEM98 suppressed the MMP-2, MMP-9, RhoC and MTA1 expression. (A and B). After 48 h of TMEM98 siRNA treatment, western Western blot was performed to identify the protein levels of MMP-2, MMP-9, RhoC and MTA1 in A549 cells. GAPDH was also detected as the control of sample loading. (C and D). After 48 h of TMEM98 siRNA treatment, the protein expressions of MMP-2, MMP-9, RhoC and MTA1 in cells were analyzed by western Western blot in H460 cells. GAPDH was also detected as the control of sample loading. **P < 0.01 compared with the control cells; ##P < 0.01 compared with the mock cells; data are expressed as the mean ± SD, n = 6.

is one of them. So far, we poorly understand the functions and the functional mechanisms of TMEM. It is predicted that TMEM98 is I type membrane molecular by TMHMM analysis. It is found that there was typical signal peptide consist of 21 amino acids in its N-terminal, which implied that TMEM98 might be a new secretory protein. It appears up-regulation under the stimulation of inflammation. This suggests that there should be some relationships between TMEM98 and the development of inflammation. It is known that a closed relationship was between inflammation and tumor [4, 5]. Many tumor progressions are accompanied with inflammatory injuries; and inflammatory microenvironment can promote the survival and the progress of tumor cells [6, 7]. Therefore, TMEM98 will be of great value for the discoveries of the new target of the treatments to inflammations and anti-tumor drugs.

Therefore, we studied the role of TMEM98 in human NSCLC. Firstly, we found that TMEM98 expressed obviously higher in lung cancer tissue than in normal tissue. Metastasis of NSCLC cancer cells is a complex multistep process including cell adhesion, invasion and migration [8, 9]. Therefore, interruption of one or more of these processes is considered as a serviceable strategy. In our study, the result indicated that siRNA-TMEM98 showed obvious inhibition of adhesion and invasion in human A549 and H460 cells. Cellular functions are regulated by multiple signal pathways and genes. The initial step of tumor cell invasion is beginning with the breakdown of the cytomembrane, a process known as dependent on type IV collagen-degrading enzymes, mainly MMP-2 and MMP-9 [10, 11]. The expression of MMPs, particularly the MMP-2 and MMP-9, has been associated with high potential of metastasis in several human carcinomas including NSCLC [12, 13]. MMP-2 and MMP-9 expression are also regulated by MTA1. Western blot analysis was employed to investigate the expression of MMP-2, MMP-9 and MTA1 in human A549 and H460 cells by siRNA-TMEM98 treatment after 48 h. Of the three Rho GTPases, RhoC is most convincingly linked with cancer metastasis. Association between RhoC and tumor progression has been described in a variety of tumor types, including breast cancer [14], non-small cell lung carcinoma [15], colon carcinoma [16], and hepatocellular carcinoma [17, 18]. In addition, our results exhibited that RhoC expression was also depressed by siRNA-TMEM98 in human A549 and H460 cells.

To conclude, we demonstrated that siRNA-TMEM98 expressed high in human NSCLC tissue and can obviously inhibit the proliferation of NSCLC cells. siRNA-TMEM98 significantly suppress the invasion and migration of human A549 and H460 cells by down regulating the protein levels of MAT1, MMP-2, MMP-9 and RhoC. Our results may also be relevant for human NSCLC carcinoma therapy.

## Disclosure of conflict of interest

None.

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