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# TRIM11 overexpression promotes proliferation, migration and invasion of lung cancer cells

Xiaolin Wang, Weiping Shi, Hongcan Shi, Shichun Lu, Kang Wang, Chao Sun, Jiansheng He, Weiguo Jin, Xiaoxia Lv, Hui Zou and Yusheng Shu\*

## Abstract

**Background:** Tripartite Motif Containing 11 (TRIM11), a member of TRIM proteins, is overexpressed in high-grade gliomas and plays an oncogenic function in glioma biology. However, little is known about the role of TRIM11 in lung cancer.

**Methods:** We analyzed TRIM11 mRNA expression in lung cancer tissues and adjacent non-neoplastic tissues by real-time PCR. We then explored the function of TRIM11 in lung cancer cells by small interfering RNA-mediated downregulation of this protein followed by analyses of cell proliferation, migration and invasion.

**Results:** TRIM11 was highly expressed in lung cancer tissues and lung cancer cell lines. The higher expression of TRIM11 was correlated with the poorer prognosis of patients. Suppressing of TRIM11 expression in lung cancer cells with higher expression of TRIM11 (A549 and NCI-H446 cells) significantly reduced cell growth, motility and invasiveness. We further demonstrated that knockdown of TRIM11 affected the expression of cell proliferation-related proteins (Cyclin D1 and PCNA), and epithelial-mesenchymal transformation-related proteins (VEGF, MMP-2, MMP-9, Twist1, Snail and E-cadherin). The activity of ERK and PI3K/AKT was also suppressed in TRIM11 knocked down cells. Further experiments in lung cells with lower expression of TRIM11 (NCI-H460 and NCI-H1975 cells) with AKT inhibitor suggested that TRIM11 may promote cell motility and invasiveness through AKT pathway.

**Conclusions:** Our results indicate that TRIM11 acts as an oncogene in lung cancer through promoting cell growth, migration and invasion. Our findings may have important implication for the detection and treatment of lung cancer.

**Keywords:** TRIM11, Lung cancer, PI3K/AKT, Migration, Invasion

## Background

Lung cancer is the most frequently diagnosed malignancy. It is the leading cause of cancer death with over 1 million death annually in the world [1]. Non-small cell lung carcinoma (NSCLC) is the most frequently occurring of lung cancer and accounts for approximately 85 % of lung cancer [1]. NSCLC includes adenocarcinoma (ADC), squamous cell carcinoma (SCC), large cell carcinoma (LCC) and others [2]. Despite recent advances in diagnosis and treatment, the prognosis of lung cancer is still poor [3]. Therefore, a better understanding of which pathways or

proteins are active in lung tumor progression will contribute to the development of early detection and targeted therapy for lung cancer [4–6].

Tripartite Motif Containing (TRIM) proteins are characterized by the presence of tripartite motif, which is composed of a RING domain, 1 or 2 B-box motifs and a coiled-coil region (RBCC) [7]. Most members of TRIM proteins, including TRIM11 could be defined as E3 ubiquitin ligases. Besides RBCC domain, TRIM11 contains a PRY domain and a SPRY domain. TRIM11 is thought to destabilize Humanin (24-amino-acid neuroprotective peptide) [8], activator-recruited cofactor 105-kDa component (ARC105) [9], PAX6 (a member of the paired-box family of transcription factors) [10] and PHOX2B (a paired box homeodomain transcription factor) [11]. Earlier studies on

\* Correspondence: shi\_wpyz@163.com

Department of Thoracic Surgery, Northern Jiangsu People's Hospital and Clinical Medical College of Yangzhou University Yangzhou, No. 98 Nantong West Road, Yangzhou 225001, People's Republic of China

TRIM11 have revealed its roles in nervous system function and development [8, 10, 11]. Recently, TRIM11 expression was found elevated in high-grade gliomas and it may exert an oncogenic function in glioma biology [12]. Other members of TRIM proteins, such as TRIM25 [13] and TRIM59 [14], have been reported to be upregulated in lung cancer, while TRIM16 [15] and TRIM31 [16] were found decreased in NSCLC. However, few investigation has been performed to test the expression and functions of TRIM11 in lung cancer.

In this study, TRIM11 expression was frequently higher in lung cancer tissues than corresponding adjacent non-neoplastic tissues. We investigated whether TRIM11 regulated cell proliferation and metastasis of lung cancer. Our study showed that TRIM11 promoted cell migration and invasion by activating the PI3K/AKT signal pathway. Our findings suggest that TRIM11 is a new potential target in lung cancer.

## Methods

### Samples

120 patients with lung cancer undergoing surgical resection at Department of Thoracic Surgery, Northern Jiangsu People's Hospital (Yangzhou, China) were enrolled in this study. The age of enrolled patients was between 34 and 72 (median 56) years. 63 participants (52.5 %) were male and 57 (47.5 %) were female. Primary lung cancer tissues were collected from all enrolled patients, while adjacent non-cancerous tissues were obtained from 35 patients of the enrolled patients. The follow-up lasted 5 years. The study was reviewed and approved by Research Ethic Committee in Northern Jiangsu People's Hospital (Yangzhou, China). Written informed consent was obtained from all patients.

### RNA isolation and real-time PCR

Total RNA from tissues and cells was extracted using Trizol (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocol. The residual DNA was removed by treating with DNase I (Roche, Indianapolis, IN, USA). Real-time PCR was used to evaluate TRIM11 mRNA levels. Total RNA (2 µg) was reverse-transcribed to cDNA with M-MLV Reverse Transcriptase Kit (Thermo Fisher, Rockford, IL, USA). The resulted cDNA was used for real-time PCR with SYBR Green qPCR Master Mix (Thermo Fisher) on ABI 7300 system (Applied Biosystem, Foster City, CA, USA) following manufacturer's instruction. The relative TRIM11 mRNA levels were calculated by normalization to GAPDH mRNA levels. The PCR primers were as follows: TRIM11 forward, 5'-CACCTAAGCTG CACAGTTCC-3'; TRIM11 reverse, 5'-GGCTGCCTCCT AATTCTTCC-3'; GAPDH forward, 5'-CACCCACTCCT CCACCTTTG-3'; GAPDH reverse, 5'-CCACCACCTG TTGCTGTAG-3'.

### Western blotting

Frozen tissue sample (about 0.1 g) was ground into powder using liquid nitrogen. Protein was extracted from frozen tissue powder and cultured cells by using RIPA lysis buffer (JRDUN Biotech., Shanghai, China) with fresh-added proteinase inhibitor cocktail (Sigma, St. Louis, MO, USA) on ice for 15 min and centrifuged at 12,000 rpm for 20 min. Protein concentrations were determined using a bicinchoninic acid kit (Thermo Fisher). For Western blotting analysis, lysates (30 µg/well) were subjected to SDS-PAGE and transferred onto nitrocellulose filter membranes. After blocking in 5 % skim milk at room temperature for 30 min, the membranes were incubated with primary antibodies overnight at 4 °C. Horseradish peroxidase conjugated secondary antibodies were subsequently used. Signals were detected using chemiluminescent substrate (ECL; Bio-Rad, Richmond, CA, USA) and exposed to X-ray film. The results were scanned and analyzed using Image J software (<http://rsb.info.nih.gov/ij/>, Bethesda, MD, USA). Antibodies against anti-TRIM11, proliferating cell nuclear antigen (PCNA), vascular endothelial growth factor (VEGF), matrix metalloproteinase (MMP)-2, MMP-9, Twist1, PI3K and p-PI3K were from Abcam (Cambridge, MA, USA). Antibodies against CyclinD1, Snail, E-cadherin (CDH1), AKT, p-AKT, ERK, p-ERK and GAPDH Cell Signaling Technology (Danvers, MA, USA).

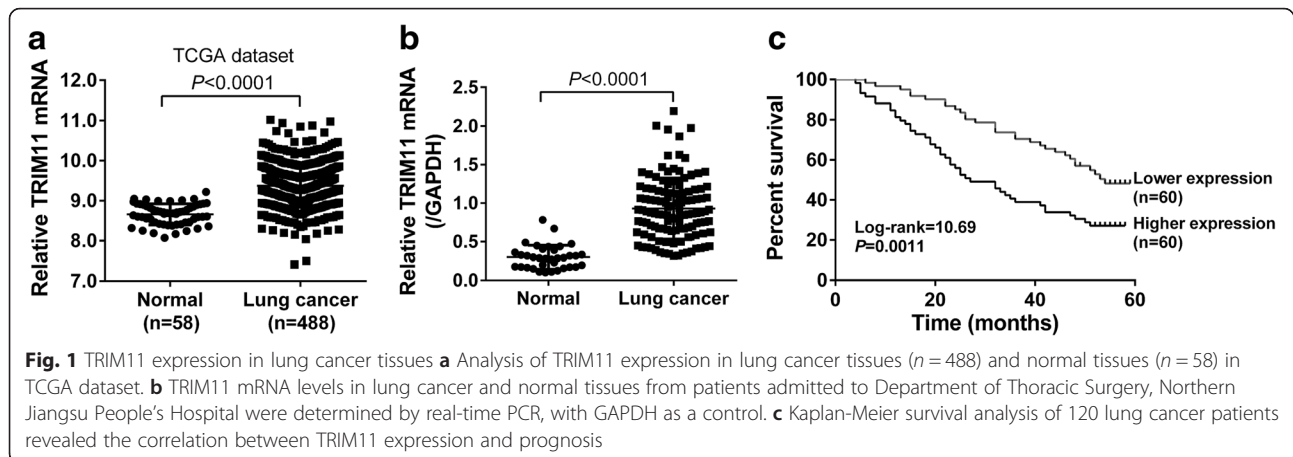
### Cell culture

Human lung adenocarcinoma cell lines A549, NCI-H1975 and PC-9, human large cell lung carcinoma cell lines NCI-H460, small cell lung cancer cell line NCI-H446 and human embryo lung fibroblasts MRC-5 were obtained from cell bank of Shanghai Biology Institute, Chinese Academy of Science (Shanghai, China). The lung cancer cell lines were cultured in RPMI 1640 medium (Invitrogen; Carlsbad, CA, USA). MRC-5 cells were maintained in Eagle's Minimum Essential Medium (EMEM). Both medium were supplemented with 10 % fetal bovine serum (FBS; Gibco, Los Angeles, CA, USA), 1 % penicillin/streptomycin and 2 mM L-glutamine. All cell lines were cultured in at 37 °C, 5 % CO<sub>2</sub>.

### Knockdown of TRIM11 expression by small interference

#### RNA (siRNA) transfection

Three siRNA targeting human TRIM11 mRNA (siRNA1, 5'-CAGAAGUUGUGCCUAUGGA-3'; siRNA2, 5'-GCU AUUACAAUUCUCGGA-3'; and siRNA3, 5'-CUAUU CAUCUUUCCCGAGA-3') and a non-specific control siRNA sequence (NC) were synthesized by Genepharma (Shanghai, China) and transfected into A549 and NCI-H446 cells with Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) per the manufacture's instruction. Real-time



PCR and Western blot analysis were performed at 48 h after transfection to assess knockdown efficiency.

#### Construction of TRIM11 lentivirus

Full-length human TRIM11 was cloned into the pLVX-AcGFP1-C1 (Clontech, Palo Alto, CA, USA). Lentiviral constructs of pLVX-AcGFP1-C1 empty vector or pLVX-AcGFP1-C1-TRIM11 were cotransfected with viral packaging plasmids (psPAX2 and pMD2.G) into 293 T cells with Lipofectamine 2000. At 48 h post transfection, viral supernatant was collected to infected NCI-H460 and NCI-1975 cells.

#### Cell proliferation assay

A proliferation assay was carried out using Cell Counting Kit-8 (CCK-8, Dojindo Laboratories, Japan) according to the manufacturer's protocol [17]. Briefly, 2000 cells/well were seeded into 96-well plates and transfected with indicated siRNA. The cells were cultured for 0, 24, 48 and 72 h, then 10  $\mu$ L CCK-8 reagent was added to each well and the cells were incubated at 37  $^{\circ}$ C for 1 h. The absorbance was recorded at 450 nm with a microplate reader (Bio-Rad).

#### Transwell migration and invasion assays

Cell migration was assayed using Transwell with 8- $\mu$ m-pore filters (Corning; New York, NY, USA) as previously described [5]. Cells were treated with the desired siRNA or expression virus. At 24 h after treatment, cells were trypsinized, resuspended in serum-free RPMI1640 medium and placed in the upper chamber ( $5 \times 10^4$  cells/well) and treated with MK-2206 (Merck, Germany) or DMSO (Sigma). Then RPMI 1640 medium supplemented with 10 % FBS was added to the lower chamber. Non-migrating cells in the upper chamber were completely removed with a cotton swab 24 h later. Migrated cells were stained with 0.5 % crystal violet and counted in five random fields.

Cell invasion assays were also performed in the same condition expect that the upper chamber was pre-coated with Matrigel (BD Biosciences, Franklin Lakes, NJ, USA).

#### Statistical analysis

All values are expressed as the mean  $\pm$  SD. Statistical analysis was tested using Graphpad Prism (Graphpad Software, San Diego, CA, USA). For comparison between

**Table 1** Correlation of TRIM11 expression with patients' features

Variables	All cases	TRIM11 mRNA		P value
		Low (n = 60)	High (n = 60)	
Age at surgery				
< 55	48	25	23	0.8523
$\geq 55$	72	35	37	
Gender				
Male	63	31	32	1.0000
Female	57	29	28	
Tumor type				
SCC	52	31	21	0.1112
ADC	34	14	20	
LCC	13	8	5	
SCLC	21	7	14	
Tumor size				
< 5 cm	49	32	17	0.0090**
$\geq 5$ cm	71	28	43	
TNM stage				
I + II	55	36	19	0.0032**
III	65	24	41	
Lymphnode metastasis				
Absent	66	41	25	0.0057**
Present	54	19	35	

Abbreviations: SCC squamous cell carcinoma, ADC adenocarcinoma, LCC large cell carcinoma, SCLC small cell lung cancer

\*\* $P < 0.01$

groups, statistical differences were tested with one-way analysis of variance (ANOVA), followed by a Sidak's test for multiple comparisons. Fisher's exact test was carried out to evaluate the relationship between TRIM11 mRNA expression and clinicopathological features. Kaplan-Meier survival curves were generated and compared by log-rank analysis.  $P < 0.05$  was considered significantly different.

## Results

### TRIM11 was highly expressed in lung cancer tissue

We re-analyzed TRIM11 expression on lung cancer cohort from The Cancer Genome Atlas project (TCGA, <https://tcga-data.nci.nih.gov/tcga/>), including 58 normal tissue samples and 488 tumor tissue samples. The results showed that TRIM11 was highly expressed in tumor tissues compared with normal tissues (Fig. 1a,  $P < 0.0001$ ).

We then detected TRIM11 mRNA levels in lung cancer ( $n = 120$ ) and adjacent normal tissues ( $n = 35$ ) by quantitative real-time PCR. As shown in Fig. 1b, TRIM11 expression was significantly higher in lung cancer tissues than that in normal tissues ( $P < 0.0001$ ).

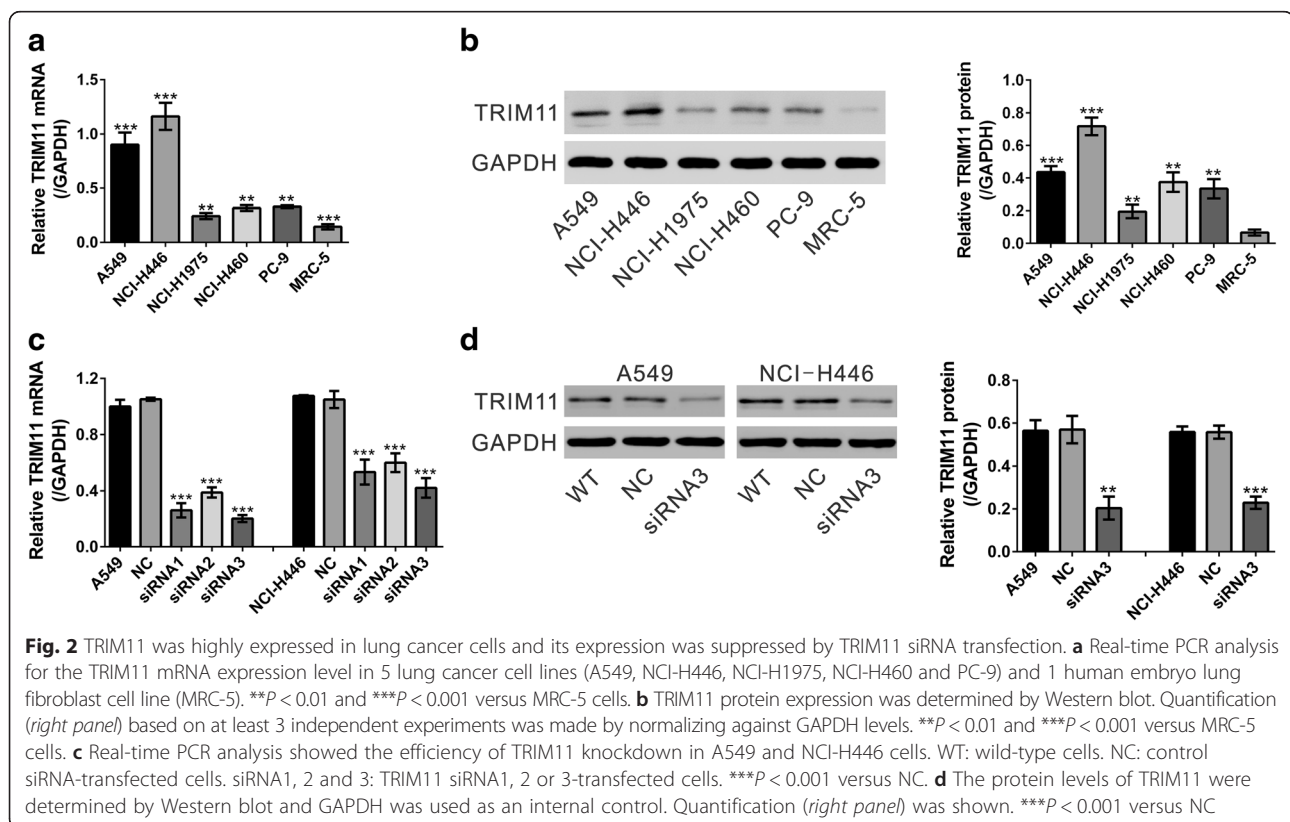
Then, according to the TRIM11 expression in tumor tissues, the 120 lung cancer patients were classified into two groups: TRIM11 higher group ( $n = 60$ ) and lower group ( $n = 60$ ). Then, the association between TRIM11 expression and patients' features was analyzed by Fisher's exact test. As shown in Table 1, TRIM11 expression

was significantly correlated with tumor size, TNM stage and lymph node metastasis. While, no correlation was observed between TRIM11 expression level and age, gender or tumor type. Kaplan-Meier analysis showed that the overall survival time of patients with higher TRIM11 expression (median survival time: 27 months) was significantly shorter than those with lower TRIM11 expression (median survival time: 54 months, Fig. 1c,  $P < 0.01$ ). Our data demonstrated that TRIM11 was over-expressed in lung cancer tissues and its expression was closely related with poor survival of patients with lung cancer.

### TRIM11 mRNA expression was inhibited in cells transfected with the TRIM11 siRNAs

TRIM11 mRNA expression was determined in 5 lung cancer cell lines (A549, NCI-H446, NCI-H1975, NCI-H460 and PC-9) and 1 human embryo lung fibroblast cell line (MRC-5). As shown in Fig. 2a, TRIM11 mRNA level was higher in most lung cancer cell lines than in MRC-5 cells. We observed similar results in the protein levels of TRIM11 (Fig. 2b).

In order to understand the functional relevance of TRIM11 expression in lung cancer progression, TRIM11 knock-down cell lines were generated by siRNA transfection. We selected two target cell lines, A549 and NCI-H446, which showed high levels of TRIM11



expression. As shown in Fig. 2c, control scrambled siRNA (NC) had no effects on TRIM11 mRNA expression compared with wild-type (WT) cells. TRIM11 siRNAs (siRNA1, siRNA2 and siRNA3) significantly inhibited TRIM11 mRNA expression compared to cells transfected with NC in both cell lines. siRNA3 had the best inhibition effect on TRIM11 mRNA expression. Western blot analysis was performed to confirm that TRIM11 protein expression was efficiently inhibited by siRNA3 transfection in both cell lines (Fig. 2d). Therefore, siRNA3 was used in all the subsequent experiments.

#### Inhibition of TRIM11 expression decreased cell proliferation

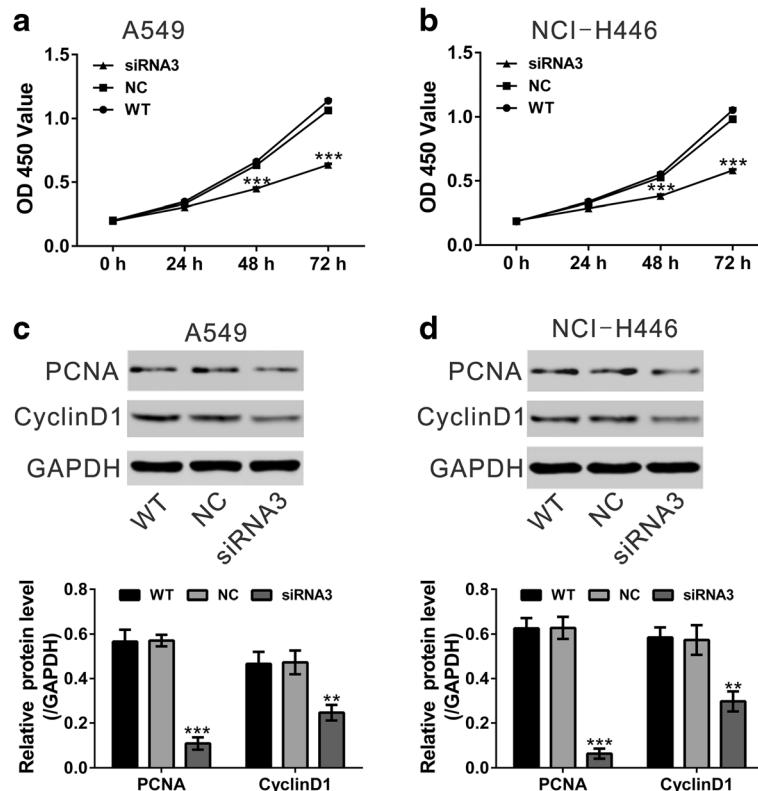
CCK-8 assay was performed to determine whether TRIM11 plays a role in the growth of lung cancer cells. Cell growth of A549, NCI-H446 (Fig. 3a and b), NCI-H460 and NCI-H1975 (Additional file 1: Figure S1) cells was obviously repressed at 48 h and 72 h after TRIM11 expression was inhibited by siRNA3 transfection. On the contrary, cell growth of two lung cancer cell lines with low levels of TRIM11 expression (NCI-H460 and NCI-H1975 cells) was significantly increased by TRIM11 overexpression (Additional file 1: Figure S2).

The protein levels of proliferation regulation proteins, PCNA and Cyclin D1, were evaluated by Western blot. The levels of PCNA and Cyclin D1 were reduced in TRIM11 knockdown cells (Fig. 3c and d). These results suggested that inhibition of TRIM11 expression inhibited cell proliferation in lung cancer cells.

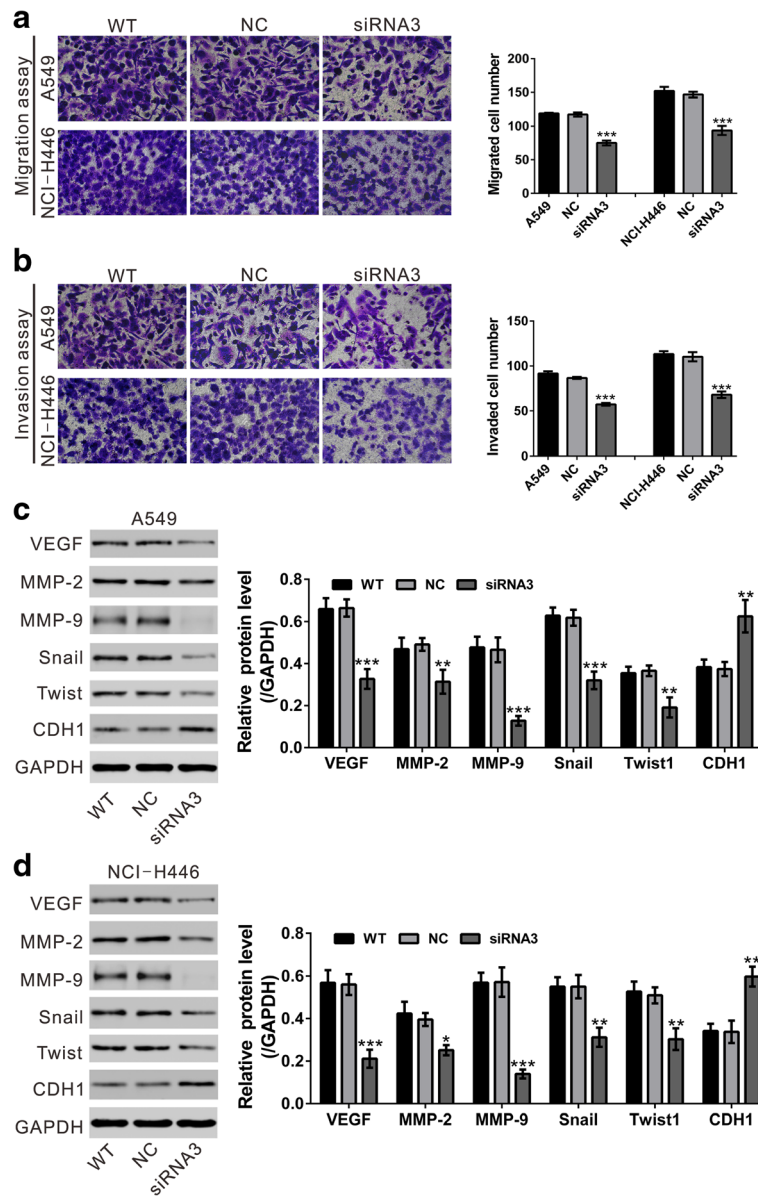
#### Suppressing of TRIM11 expression inhibited the motility and invasiveness of lung cancer cells

The ability of cancer cells to undergo migration and invasion allows them to spread with the tissues and metastasize to distant organs [18]. To investigate the function of TRIM11 in the metastasis, the effects of TRIM11 on the migrated and invasive ability of lung cancer cells were assessed by Transwell assay. As shown in Fig. 4a and b, silencing of TRIM11 in A549 and NCI-H446 cells caused a significant reduction in cell migration and cell invasion.

Moreover, epithelial-mesenchymal transformation (EMT) is critical for metastasis of cancer cells [19]. We then detected the protein levels of metastasis and EMT related proteins by Western blot (Fig 4c and d). The protein levels of VEGF, MMP-2, MMP-9, Snail and Twist1 were significantly down-regulated in A549 and NCI-H446



**Fig. 3** Knockdown of TRIM11 reduced the proliferation of lung cancer cells. **a, b** CCK-8 assay results of A549 and NCI-H446 cells at 0 h, 24 h, 48 h and 72 h after TRIM11 expression was inhibited by siRNA3 transfection. **c, d** Expression of PCNA and Cyclin D1 was evaluated by Western blot. Quantification (bottom panel) based on at least 3 independent experiments was shown. \*\*\* $P < 0.001$  versus NC



**Fig. 4** Knockdown of TRIM11 inhibited the motility and invasiveness of lung cancer cells. **a** Migration assay was performed on wild-type (WT) cells, cells transfected with control siRNA (NC) and cells transfected with TRIM11 siRNA3 (siRNA3). Representative images (*left panel*) and quantitative results (*right panel*) were shown. **b** Invasion assay was carried out. **c, d** Expression of metastasis-related proteins was evaluated by Western blot. **e** \* $P < 0.05$ , \*\* $P < 0.01$  and \*\*\* $P < 0.001$  versus NC

cells with TRIM11 knockdown, while the main factor of EMT, E-cadherin (CDH1) was significantly increased in TRIM11 knockdown cells. These data suggested an inhibitory role of TRIM11 on EMT and lung cancer metastasis.

#### Silencing of TRIM11 repressed the activation of AKT pathway in lung cancer cells

Extracellular signal-regulated kinases (ERKs) [20] and PI3K/AKT [21] pathways, which are key signaling pathways involved in cell growth and migration, are frequently overactivated in tumor cells. Knockdown of TRIM11 in

glioblastoma multiforme cells suppressed the activity of ERK pathway, while had no effects on PI3K/AKT activity [12]. To explore the effect of TRIM11 on PI3K/AKT and ERK pathways in lung cancer cells, phosphorylation of PI3K, AKT and ERK was determined by Western blot. As shown in Fig. 5, transfection of TRIM11 siRNA in lung cancer cells significantly reduced the phosphorylation of PI3K, AKT and ERK.

To further confirm the involvement of PI3K/AKT signaling, an AKT inhibitor (MK-2206) was used. NCI-H460 and NCI-H1975 cells, which showed low levels of

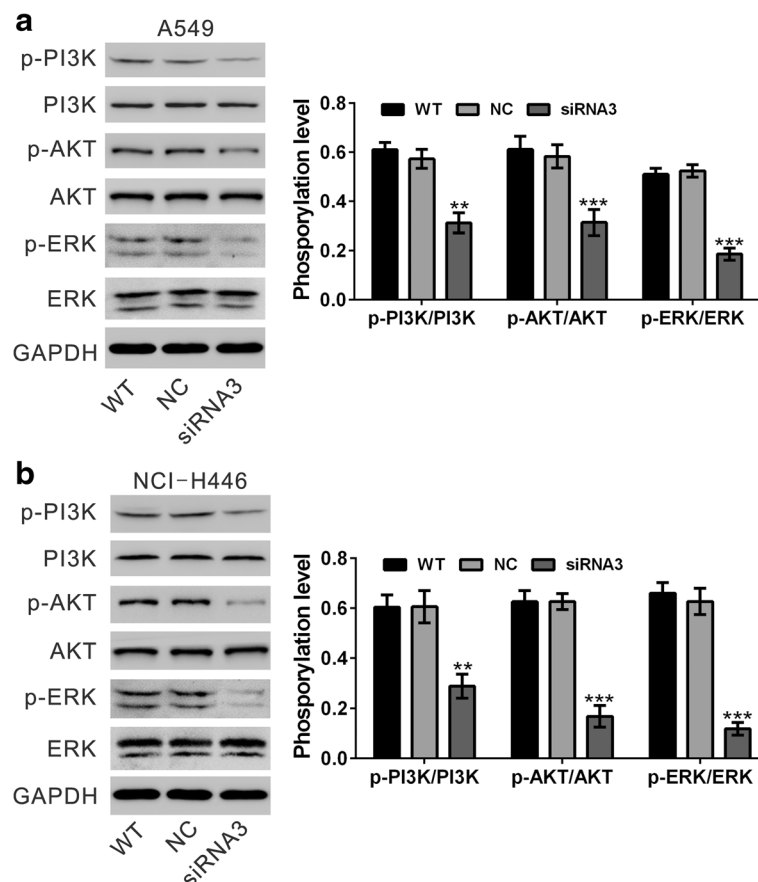
TRIM11 expression, was overexpressed with TRIM11 and treated with MK-2206. Cell migration and invasion was then evaluated by Transwell assays (Fig. 6). Cell migration and invasion of lung cells was significantly enhanced by TRIM11 overexpression, but suppressed by MK-2206 treatment. More importantly, the promotion effects of TRIM11 overexpression on cell migration and invasion were weakened by MK-2206 exposure. These data indicated that TRIM11 may promote cell migration and invasion partially by activating PI3K/AKT pathway.

## Discussion

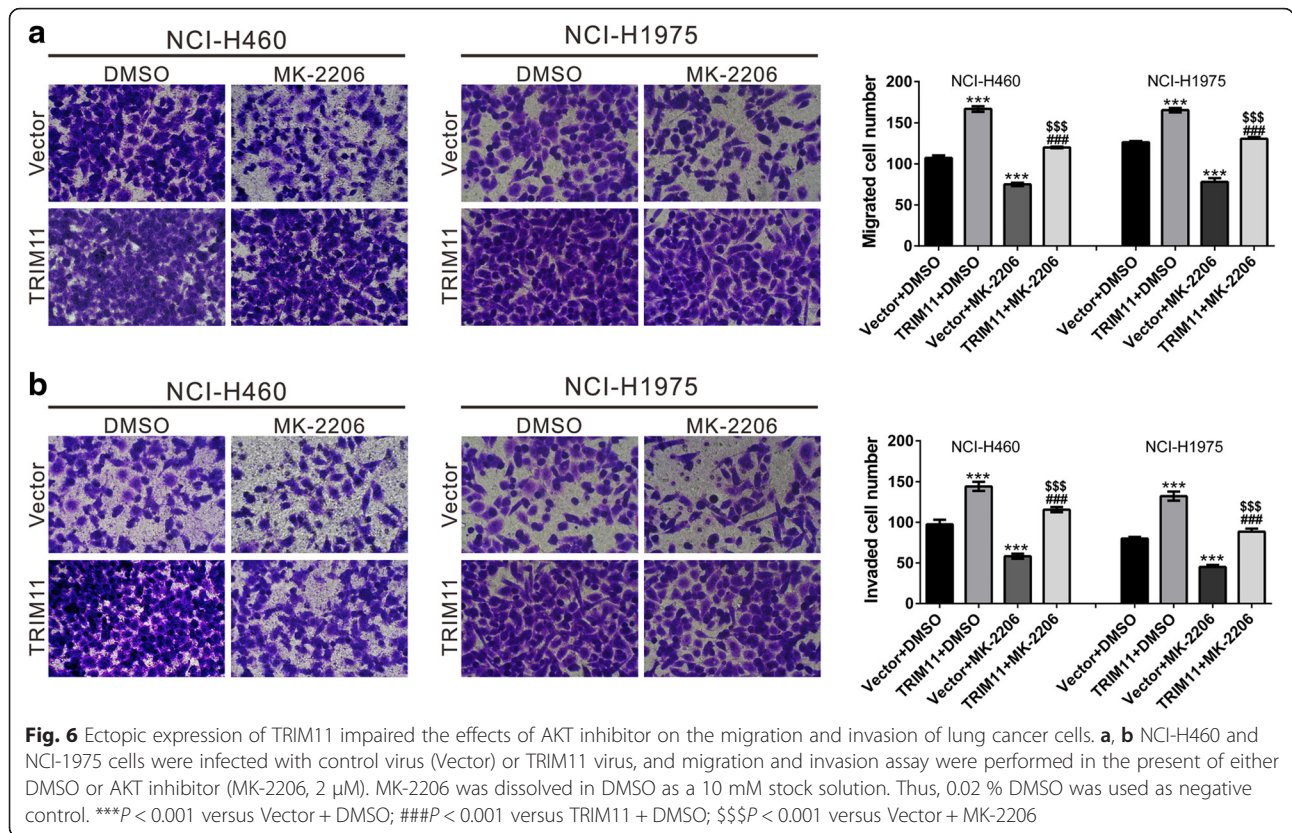
The altered expression of TRIM proteins has been reported in a variety of human cancers, including lung cancer [13–16]. Di, K. et al. demonstrated diagnostic and prognostic value of TRIM11 in gliomas [12]. However, the expression and function of TRIM11 in lung cancer has been poorly characterized. Here, by analyzing on-line available dataset (Fig. 1a), we found that TRIM11 mRNA was elevated in lung cancer tissues, which was confirmed by real-time PCR analysis on 120 patients admitted at Department of Thoracic Surgery, Northern

Jiangsu People's Hospital (Fig. 1b). Fisher's exact tests (Table 1) and Kaplan-Meier survival curves (Fig. 1c) demonstrated the strong association between TRIM11 expression and tumor size, TNM stage, lymph node metastasis, as well as overall survival of lung cancer patients. Thus, TRIM11 may serve as a useful diagnosis and prognosis marker for lung cancer although further in-depth clinical study is needed.

Then we investigated the functions of TRIM11 in lung cancer. The in vitro experiments demonstrated that inhibition of TRIM11 expression in lung cancer cells with higher expression of TRIM11 (A549 and NCI-H446 cells) suppressed cell growth (Fig. 3), migration (Fig. 4a) and invasion (Fig. 4b). On the contrary, ectopic expression of TRIM11 in lung cells with lower expression of TRIM11 (NCI-H460 and NCI-H1975 cells) had inverse effects (Additional file 1: Figure S2 and Fig. 6). These data suggest that TRIM11 may play an oncogenic role by promoting the proliferation, migration and invasion of lung cancer cells. Our results of in vitro experiments were consistent to our clinical observation that high expression of TRIM11 associated with lymph node



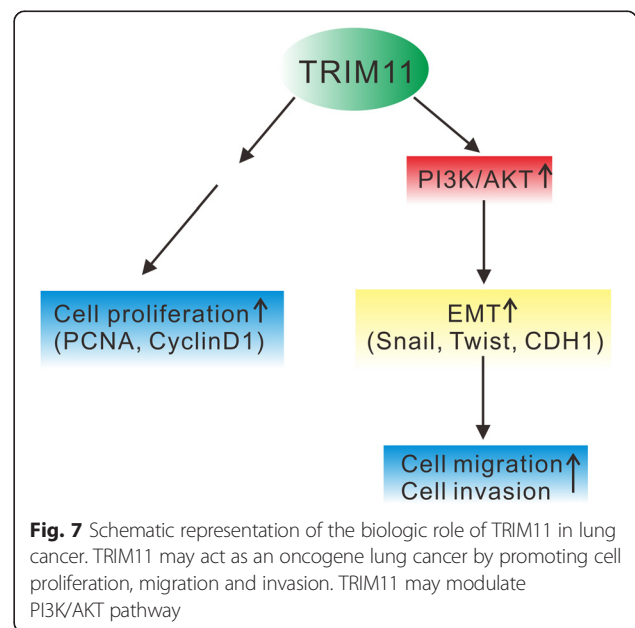
**Fig. 5** Knockdown of TRIM11 inhibited the activation of PI3K/AKT and ERK pathways in lung cancer cells. Total protein and phosphorylation of PI3K, AKT and ERK was evaluated by western blot in A549 **a** and NCI-H446 cells **b**. \*\* $P < 0.01$  and \*\*\* $P < 0.001$  versus NC



metastasis and tumor size. Furthermore, cancer cells gain migratory and invasive properties through the process of EMT [22]. Functional loss of E-cadherin has been considered as a key event during the process of EMT [23]. Twist1 and Snail can repress the expression of E-cadherin, and has been regarded as EMT-inducers [24]. VEGF [25] and MMP-2/9 [26] are well-known to play an important role in tumor metastasis. Here, knock-down of TRIM11 decreased the expression of metastasis (VEGF, MMP-2 and MMP-9) and EMT-related proteins (Twist1 and Snail), while increased the expression of E-cadherin. Our data suggested that TRIM11 siRNA may inhibit lung cell invasion via suppressing EMT.

Further, in addition to cellular phenotype, we tried to investigate the molecular mechanism through which TRIM11 works as an oncogene in lung cancer. ERK and PI3K/AKT pathways can promote cell growth and metastasis [27, 28]. Both ERK [20] and PI3K/AKT [21] pathways are frequently over-activated in various tumor types. Di, K. et al. found that knockdown of TRIM11 in glioblastoma multiforme cells had no effects on PI3K/AKT activity, but suppressed ERK activity [12]. In the current study, we found a notable decrease of ERK and PI3K/AKT activation (Fig. 5) in TRIM11 knocked down lung cancer cells. The discrepancy of data and the previous study [12] could be due to the different cell types used. Accumulating evidence points to the important role of cell motility and

invasion in cancer progression and metastasis [29]. Phosphorylation levels of AKT was found to be associated with the invasion and metastasis of NSCLC [30]. In the present study, TRIM11 overexpression can promote the PI3K/AKT pathway (Additional file 1: Figure S2). The promotion effects of TRIM11 overexpression on cell migration





and invasion were significantly weakened by the exposure of AKT inhibitor, MK-2206 (Fig. 6), which suggested that TRIM11 might promote cell migration and invasion partially by activating AKT pathway. Thus, we speculated that elevated TRIM11 will facilitate the development and invasive property of lung cancer through AKT pathways. However, the mechanisms are needed to be further elucidated in detail.

## Conclusions

In summary, increased expression of TRIM11 was noted in lung cancer tissues. TRIM11 functions as an oncogene in lung cancer through promoting cell proliferation, migration and invasion. Moreover, we demonstrated that TRIM11 may modulate PI3K/AKT pathway (Fig. 7). Considering that TRIM11 expression level was associated with patients' overall survival, TRIM11 may be a new potential target in lung cancer treatment.

## Additional file

**Additional file 1: Figure S1.** Knockdown of TRIM11 reduced the proliferation of NCI-H460 and NCI-H1975 cells. **Figure S2.** Effects of TRIM11 overexpression on cell proliferation. (DOCX 884 kb)

## Abbreviations

ARC105, activator-recruited cofactor 105-kDa component; CCK-8, Cell Counting Kit-8; EMEM, Eagle's Minimum Essential Medium; EMT, epithelial-mesenchymal transformation; ERKs, extracellular signal-regulated kinases; FBS, fetal bovine serum; MMP, matrix metalloproteinase; NSCLC, non-small cell lung carcinoma; PCNA, proliferating cell nuclear antigen; TCGA, The Cancer Genome Atlas project; TIRM, Tripartite Motif Containing; VEGF, vascular endothelial growth factor.

## Acknowledgments

Not applicable.

## Authors' contributions

Conception and design: XLW, YSS; Performed the experiments: XLW, WPS, HCS, SCL; Analysis and interpretation of data: XLW, KW, CS, JSH; Contributed reagents/materials: WGJ, XXL, HZ; Wrote the paper: XLW, WPS, YSS. All authors have read and approved the final manuscript.

## Competing interests

The authors declare that they have no competing interests.

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