PTTG1 promotes migration and invasion of human non-small cell lung cancer cells and is modulated by miR-186

Hongli Li1,2,†, Chonggao Yin3,†, Baogang Zhang1,4,†, Yonghong Sun1, Lihong Shi1, Ningbo Liu3, Shujuan Li3, Shijun Lu1,†, Yuqing Liu1,†, Jin Zhang2,†, Fengjie Li1,†, Wentong Li1,†, Fei Liu1,†, Lei Sun1,† and Yuliang Qi1,†

†Department of Pathology, Weifang Medical University, Weifang 261053, China, 2Department of Oncology, Weifang Medical University, Weifang 261053, China, 3Department of Pathology, Key Clinical Specialty for Pathology of Shandong Province, Affiliated Hospital of Weifang Medical University, Weifang 261053, China, 4Department of Pharmacology, Weifang Medical University, Weifang 261053, China, 5Department of Radiation Oncology, Tianjin Medical University Cancer Institute and Hospital, Tianjin 300192, China and 6Key Laboratory for Immunology in Universities of Shandong Province Weifang Medical University, Weifang 261053, China

*To whom correspondence should be addressed. Tel: +86 536 8462034; Fax: +86 536 8462034; Email: zhangbg@wfmc.edu.cn or zbg0903@hotmail.com

Deeper mechanistic understanding of non-small cell lung cancer (NSCLC), a leading cause of total cancer-related deaths, may facilitate the establishment of more effective therapeutic strategies. In this study, pituitary tumor transforming gene (PTTG1) expression was associated with lymph node and distant metastasis in patients with NSCLC and was correlated with patient survival. Reduction of PTTG1 by small interfering RNA (siRNA) inhibits migration and invasion of NSCLC cells by modulating metalloproteinases expression. To the best of our knowledge, this study is the first to report that PTTG1 promotes epithelial growth factor (EGF) induced the phosphorylation of LIN-11, Isl1 and MEC-3 protein domain kinase and cofilin, a critical step in cofilin recycling and actin polymerization. Additionally, EGF-induced Akt phosphorylation was suppressed through knockdown of PTTG1. Interestingly, miR-186 can modulate PTTG1 protein expression. As observed from the animal experiment in this study, knockdown of PTTG1 through siRNA and overexpression of miR-186 inhibited invasive activity of NSCLC cells toward the SCID mice lung. In summary, our in vitro and in vivo results indicate that PTTG1 modulated by miR-186 has an important function in NSCLC invasion/metastasis. This study identified both PTTG1 and miR-186 as potential anti-invasion targets for therapeutic intervention in NSCLC.

Introduction

Lung cancer is the leading cause of cancer death worldwide, accounting for 18% of cases in the 2008 global statistics (1). The two major types of lung cancer are non-small cell lung cancer (NSCLC) and small cell lung cancer. NSCLC accounts for 75–85% of the cases and is further classified as adenocarcinoma (40%), squamous cell carcinoma (30–40%) and large cell carcinoma (5–15%) (2). Metastasis is the main factor contributing to mortality in patients with lung cancer. Metastasis is a multistep process wherein cancer cells are disseminated from the primary tumor, locally invading the surrounding tissue where they enter blood vessels (intravasation) and subsequently exit the bloodstream (extravasation), thus colonizing the new microenvironment and forming secondary tumors (3). Numerous molecular markers, such as cell cycle regulators, cell adhesion proteins and growth factors, have recently been investigated in relation to carcinoma metastasis.

The protein product of the pituitary tumor transforming gene (PTTG) was first isolated from GH4 rat pituitary tumor cells (4,5) and shown to be transforming in vitro and tumorigenic in vivo (6). The PTTG family includes PTTG1, PTTG2 and PTTG3. PTTG1 is the most abundant and widely studied form of the substance and is referred to as PTTG (7). A 60% reduction in PTTG protein using small interfering RNA (siRNA) in the lung cancer cell line H1299 showed inhibited colony formation using soft agar assay and reduced xenograft tumor formation in nude mice (2), furthermore demonstrating the function of PTTG1 in tumor growth and progression. Previous research showed that PTTG induced EMT through integrin α4 and β3-focal adhesion kinase signaling in lung cancer cells (8) and that PTTG1 promotes tumor malignancy via epithelial to mesenchymal transition and expansion of breast cancer stem cell population (9). However, whether PTTG1 has any function in the migration and invasion of NSCLC cells through cytoskeleton rearrangement remains unknown.

MicroRNAs (miRNAs) are evolutionarily conserved, small non-coding RNA molecules that negatively regulate gene expression at the post-transcriptional level, binding through partial sequence homology to the 3′-untranslated region (3′-UTR) of target messenger RNAs (mRNAs) and causing translational inhibition and/or mRNA degradation (10). Several studies have documented numerous abnormal miRNA expression patterns in diverse human malignancies, with miRNAs acting as oncogenes, whereas others as tumor suppressor genes. These observations suggest that the function of different miRNAs in carcinogenesis may vary, thus requiring careful examination (11). More recently, miR-126, miR-335 and miR-206 have been shown to function as metastasis suppressor miRNAs in human breast cancer (12), and miR-126 has been proven to affect breast cancer and lung cancer invasion (13). Previous studies showed that miR-186 downregulation correlates with poor survival in NSCLC and that it interferes with cell cycle regulation. However, whether miR-186 modulates PTTG1 in NSCLC invasion remains poorly understood.

In this study, we determined the correlation of PTTG1 expression in NSCLC tissue specimens with the clinicopathologic characteristics of patients and then examined the effect of PTTG1 expression on NSCLC migration and invasion. To the best of our knowledge, this study is the first to report that PTTG1 promotes the migration and chemotaxis of NSCLC cells. Mechanistically, PTTG1 is an important factor in cofilin recycling and actin polymerization. Functional assays demonstrated that miR-186 inhibits the invasion and metastasis of NSCLC cells by modulating PTTG1.

Materials and methods

Patients and tissue specimens

Paraffin-embedded specimens were collected from 146 patients who had undergone surgical treatment for primary lung cancer with pathologic identification in the Affiliated Hospital of Weifang Medical University and the Weifang People’s Second Hospital from 2001 to 2007, guided by a protocol approved by the institutional review board. Patients gave consent for the use of their tissue specimens in this study. None of them had received chemotherapy or radiotherapy before surgery. The histological characterization and clinicopathological staging of the samples were determined according to the 2009 International Union Against Cancer Tumor-Node-Metastasis classification. A total of 107 males and 39 females were included in this study, ranging in age from 38 to 76 years (median age 55 years). Clinical information of the samples is described in detail in Supplementary Table 1, available at...
Carcinogenesis Online. Twenty paired NSCLC and adjacent non-tumor (ANT) were selected for western blot.

Immunohistochemistry and evaluation criteria

Immunohistochemical analysis was done to study altered protein expression in all human NSCLCs using utilizing streptavidin-peroxidase assay following the manufacturer’s instructions. Primary antibodies used for immunostaining were the PTTG1 polyclonal (Santa Cruz Biotechnology; 1:500 dilution). Negative controls (NCs) were used whereby the primary antibody was omitted. The degree of immunostaining of formalin-fixed, paraffin-embedded sections was reviewed and scored independently by two observers, based on both the proportion of positively stained tumor cells and the intensity of staining (14). The proportion of positively stained tumor cells was graded as follows: 0 (no positive tumor cells), 1 (<10% positive tumor cells), 2 (10–50% positive tumor cells) and 3 (>50% positive tumor cells). The cells at each intensity of staining were recorded on a scale of 0 (no staining), 1 (weak staining, light yellow), 2 (moderate staining, yellowish brown) and 3 (strong staining, brown). The staining index was calculated as follows: staining index = staining intensity × proportion of positively stained tumor cells. Using this method of assessment, we evaluated the expression of PTTG1 by staining index (scored as 0–4, 6 or 9). The staining index scores 6 and 9 were used to define tumors with strong expression, 1–4 as tumors with weak expression and 0 to 0 as tumors with no expression. Scores 0–4 were regarded as low expression; 6 and 9 were regarded as high expression.

Cell lines

The BEAS-2B immortalized human bronchial epithelial cell line was cultured in LHC-9 medium. The human NSCLC cell lines A549, SPC-A1, Calu1 and H1299 were obtained from American Type Culture Collection (ATCC, Manassas, VA) and grown in Dulbecco’s modified Eagle’s minimal essential medium and RPMI-1640, respectively, supplemented with 10% fetal bovine serum, at 37°C in a humidified atmosphere of 95% air and 5% CO₂.

Plasmid construction and cell transfection

MiR-186 mimics, NC mimics and PTTG1 siRNAs were synthesized by GenePharma (Shanghai, China). For H1299 cells, 2 × 10⁴ cells were plated in 35 mm dish for 24 h before transfection in complete medium. Transfection was performed with Lipofectamine 2000 (Invitrogen) according to the manufacturer’s instructions. For transient transfections, the siRNAs include PTTG1 siRNA duplex no. 1: 5′-GACCCUGGAUGUUGAAUUG-3′ and duplex no. 2: 5′-GAGAAGAGCUUGAAAGCATT-3′ and a scrambled siRNA was synthesized by Invitrogen. For stable transfection, two siRNA expression plasmid containing a target sequence and a vector containing a scrambled sequence were obtained from Genchem Co. Stable transfectants were selected by using 700 µg/ml neomycin.

PTTG1 complementary DNA (cDNA) was cloned in the KpnI-XbaI sites of pcDNA3.1 (Invitrogen) and was confirmed by DNA sequencing. A549 cells were transfected with pcDNA3.1-PTTG1 plasmid or pcDNA3.1 vector using Lipofectamine 2000 (Invitrogen) following the protocol. After 24 h of transfection, cells were trypsinized, diluted and seeded into 10 cm culture dishes. Stable transfected cells were obtained by selection medium (culture medium with 600 µg/ml G418). Single cell clones were isolated for clone expansion. Stable transfected cell clones were maintained and passaged in culture medium with G418 (300 µg/ml). 3′-UTRs of PTTG1 were amplified and then cloned into the downstream of the luciferase gene in a modified pGL3 control vector (Promega, Madison, WI).

A DNA fragment containing the hsa-miR-186 precursor with 300 bp flanking sequence of each side was amplified into retroviral transfer plasmid pMSCV-puro (GenePharma). Retroviral production and infection were performed as described previously. Following transduction, puromycin (1.5 µg/ml) was used as a selection antibiotic to select the infected cells for 10 days.

RNA extraction and quantitative real-time–PCR

Total miRNA of cultured cells, surgically resected NSCLC tissues and paraffin-embedded, archival clinical NSCLC specimens was extracted using the mirVana miRNA Isolation Kit (Ambion, Austin, TX) and RecoverAll™ Total Nucleic Acid Isolation Kit (Ambion) according to the manufacturer’s instructions. cDNA was synthesized with 5 ng of total RNA using the TaqMan™ miRNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA), and the expression levels of miR-186 were quantified with the miRNA-specific TaqMan™ MiRNA Assay Kit (Applied Biosystems) (15). U6 snRNA levels were used for normalization.

Western blot

For western blot, cells or tissues were directly lysed in 1× sodium dodecyl sulfate sample buffer. Cells were stimulated with 10 ng/ml epidermal growth factor (EGF) in serum-free medium for 0, 30 and 90 min and phosphorylation of Akt and LIN-11, Isl1 and MEC-3 protein domain kinase (LIMK)/cofilin was analyzed. Following antibodies were used: anti-PTTG1 (Santa Cruz Biotechnology; 1:1000), p-Akt (Ser-473; Cell Signaling; 1:1000), p-Akt (Thr-308; Cell Signaling; 1:1000), p-LIMK/cofilin (Cell Signaling; 1:1000), Akt (Cell Signaling; 1:1000), LIMK/cofilin (Cell Signaling; 1:1000), β-actin (Cell Signaling; 1:1000) and horseradish peroxidase-linked anti-rabbit IgG antibody (Cell Signaling; 1:2000). All experiments were repeated at least three times.

Chemotaxis assay

Chemotaxis assays were performed by using transwell chambers as described previously (17,18). Briefly, the chemoattractant (EGF) was loaded into the lower chemotaxis chamber and 5 × 10⁵ cells/ml suspended in the binding medium (RPMI 1640, 0.1% bovine serum albumin and 25 mM N-2-hydroxypiperazine-N′-2-ethanesulfonic acid (HEPES)) were added to the upper chamber. The polycarbonate filter (8 µm pore size) was pretreated with 10 µg/ml fibronectin overnight, dried in air and inserted between the upper and lower chambers. Then, the chamber was incubated at 37°C in 5% CO₂ for 3 h. The filter membrane was then rinsed, fixed and stained. The numbers of migrating cells were counted at ×400 in three separate fields by light microscope.

Cellular F-actin measurement

The F-actin content was detected as described previously (17,18). Briefly, the cells were suspended at 4 × 10⁵/ml in medium supplemented with 10% fetal bovine serum albumin, 10 mM HEPES (pH 7.2) and preincubated for 3 h at 37°C. The cells were followed by the stimulation of 10 ng/ml EGF at 37°C at different time points. The cells were then fixed with 4% paraformaldehyde for 10 min, permeabilized with 0.1% Triton X-100 for 30 min and incubated with Alexa-fluor 568 phalloidin in F-actin buffer (10 µmol/l HEPES, 20 µmol/l KH₂PO₄, 5 mmol/l ethylene glycol-bis(aminohexy)tetraacetic acid, 2 mmol/l MgCl₂, phosphate-buffered saline, pH 7.4) at room temperature for 60 min. The cells were washed five times. F-actin content was measured with a microplate fluorescence reader with an excitation wavelength of 578 nm and an emission wavelength of 600 nm. The relative F-actin content over different time periods was calculated by the following equation: F-actin ΔF/F-actin 0 = fluorescence Δfluorescence 0.

Matrigel invasion assay

A Boyden chamber invasion assay was performed as described previously (19). Briefly, the transwell inserts with 8 μm pore size were coated with a final concentration of 1.5 mg/ml of matrigel. The cells were suspended in serum-free medium at a final concentration of 4 × 10⁵/ml and incubated at 37°C for 30 min. About 300 µl of binding medium (RPMI-1640, 0.1% bovine serum albumin and 25 mM HEPES) with 10 ng/ml of insulin-like growth factor-1 was added to the lower well. After 24 h of incubation (37°C, 5% CO₂), the non-invading cells were removed by wiping the upper side of the membrane, and the invading cells were fixed and stained. The number of invading cells was counted under a microscope (Olympus, IX71) in five predetermined fields (CellSens Standard; total magnification ×200). All assays were performed at least three times independently.

Enzyme-linked immunosorbent assay

Enzyme-linked immunosorbent assay (ELISA) was performed as described previously (20). Cells (2 × 10⁵ cells per well each) were plated onto six-well plates in Dulbecco’s modified Eagle’s minimal essential medium and RPMI-1640 containing 5% fetal bovine serum albumin and 25 mM HEPES. After 24 h of incubation (37°C, 5% CO₂), the non-invading cells were removed by wiping the upper side of the membrane, and the invading cells were fixed and stained. The number of invading cells was counted under a microscope (Olympus, IX71) in five predetermined fields (CellSens Standard; total magnification ×200). All assays were performed at least three times independently.

Gelatin zymography

Gelatin zymography was performed as described previously (21,22). Cells transfected with control or PTTG1 siRNA were treated with 10 ng/ml EGF for 12 h in serum-free medium. The conditioned medium was collected and analyzed on 10% sodium dodecyl sulfate–polyacrylamide gel incorporated with 0.1% gelatin.

Luciferase reporter assay

Cells (3.5 × 10⁴) were seeded in triplicates in 48-well plates and allowed to settle for 24 h. About 100 ng of pGL3-PTTG1-3’-UTR plus 1 ng of pRL-TK renilla plasmid (Promega) were transfected using the Lipofectamine 2000
PTTG1 modulated by miR-186 regulates NSCLC invasion

These chemotaxis assay results indicate that PTTG1 has an important function in the EGF-induced chemotaxis of NSCLC cells.

Reduction of PTTG1 inhibited EGF-induced F-actin polymerization in NSCLC cells

One of the mechanisms for cell migration toward the leading edge during migration is the polymerization of F-actin toward the cell membrane. The ligand-induced transient F-actin assembly correlates with cellular chemotactic capacity (25,26). To prove that the reduction of PTTG1 could inhibit the EGF-induced chemotaxis of H1299 cells by inhibiting F-actin polymerization, we performed an F-actin polymerization assay. The F-actin fluorescence assay results showed that EGF induced an increase in F-actin content in Scr/H1299 cells, but not in SiPTTG1/H1299 cells (Figure 1D). The doubling time of SiPTTG1/H1299 cells did not differ from that of Scr/H1299 cells (Figure 2B). Chemotaxis assay showed similar results between parental H1299 and Scr/H1299 cells. The SiPTTG1/H1299 cells showed decreased chemotaxis compared with Scr/H1299 cells (Figure 2C).

Meanwhile, PTTG1 stably transfected cell clones were generated through transfection with pcDNA3.1-PTTG1 plasmid and subsequent selection. Thus, we chose to present the results from clone 2, designated as A549/PTTG1 cells, as the representative. A549 cells were also transfected with a pcDNA3.1 vector to establish vector control cells, which were designated as A549/Con. The identification of PTTG1 stably transfected cell clones by western blot analysis is illustrated in Figure 2D. The rate of cell proliferation was also examined in both A549/PTTG1 and A549/Con cells in vitro. The doubling time of A549/PTTG1 cells did not differ from that of A549/Con cells (Figure 2E). The A549/PTTG1 cells showed increased chemotaxis compared with the A549/Con cells (Figure 2F). These chemotaxis results indicate that PTTG1 has an important function in the EGF-induced chemotaxis of NSCLC cells.

Results

Upregulation of PTTG1 in NSCLC cell lines and NSCLC tissues as well as association between PTTG1 and clinicopathological features of NSCLC

Comparative evaluation of 20 paired NSCLC and ANT tissues by western blot analysis, with each pair obtained from the same patient, was performed to detect lung micrometastasis. The lung tissues were fixed with formalin and embedded in paraffin for immunohistochemical evaluation. The lung tumor xenografts were harvested for further evaluation. The lung tissues were fixed with formalin and embedded in paraffin to examine metastasis. Serial sections and hematoxylin and eosin staining were performed to detect lung micrometastasis.

Statistical analysis

A cohort of 146 NSCLC patients was divided into two groups based on miR-186 expression level for clinical survival analysis: the low-miR-186 expression group (below the median value) and the high-miR-186 expression group (above the median value). All statistical analyses were performed using the SPSS 16.0 statistical software package. The chi-square test was used to analyze the relationship among miR-186, PTTG1 expression and clinicopathologic characteristics. Survival curve was plotted using the Kaplan–Meier method and was compared using the log-rank test. Statistical significance for comparisons between groups was determined using Student's paired two-tailed t-test or analysis of variance (ANOVA). P < 0.05 was considered statistically significant in all cases.

Association between PTTG1 expression and patient prognosis

Kaplan–Meier analysis using the log-rank test was performed, and the result demonstrated that patients with low PTTG1 expression in their lung tumors had a longer median survival time of 28 months compared with those with high PTTG1 expression, whose median survival time was 15 months (P < 0.001; Figure 1C). Moreover, we found that patients with tumors exhibiting high PTTG1 expression exhibited significantly shorter overall survival compared with those having low PTTG1 expression in either the stage I and II subgroup (n = 88; P = 0.001, log-rank; Figure 1D) or the stage III and IV subgroup (n = 58; P = 0.042, log-rank; Figure 1E). Thus, high PTTG1 expression appears to be a risk factor predicting poor survival, suggesting that increased PTTG1 expression likely contributes to NSCLC pathogenesis and might represent a prognostic biomarker for the disease.

PTTG1 promotes NSCLC cells migration

To determine the specific involvement of PTTG1 in the migration of H1299 cells, we applied siRNA technology to inhibit PTTG1 expression. Transfected cells were selected through hygromycin B resistance to establish cell lines that stably downregulated PTTG1 expression. A number of stable clones were obtained using these methods. The siRNAs include PTTG1 siRNA duplex no. 2, which was stably transfected, and similar phenotypes were also obtained (data not shown). Two representative clones (clones 1 and 2) were used in the analysis (Figure 2A). Given that these clones had similar phenotypes, we chose to present the results from clone 1, designated as SiPTTG1/H1299 cells, as the representative. An siRNA construct containing a scrambled sequence was transfected into H1299 cells to generate control cells, which were designated as Scr/H1299 cells. The rate of cell proliferation was examined in both Scr/H1299 and SiPTTG1/H1299 cells in vitro. The doubling time of SiPTTG1/H1299 cells did not differ from that of Scr/H1299 cells (Figure 2B). Chemotaxis assay showed similar results between parental H1299 and Scr/H1299 cells. The SiPTTG1/H1299 cells showed decreased chemotaxis compared with Scr/H1299 cells (Figure 2C).

We used immunohistochemical analysis to determine PTTG1 expression in NSCLC specimens. The percentage of positive tumor cells and the staining intensity for each sample were recorded. The positive expression rate of the PTTG1 was 84.9% (124/146) in NSCLC cases and 11.6% (17/146) in para-neoplastic tissues. A significant statistical difference was found between the two groups (P < 0.001; Figure 1B). The correlations between PTTG1 expression and NSCLC clinical pathologic features are summarized in Supplementary Table 1, available at Carcinogenesis Online. The level of PTTG1 expression closely correlated with NSCLC clinical staging (P < 0.001) as well as N classification and distant metastasis of Tumor-Node-Metastasis classification (P < 0.001 and P = 0.026, respectively; Figure 1B).

Association between PTTG1 expression and patient prognosis

Kaplan–Meier analysis using the log-rank test was performed, and the result demonstrated that patients with low PTTG1 expression in their lung tumors had a longer median survival time of 28 months compared with those with high PTTG1 expression, whose median survival time was 15 months (P < 0.001; Figure 1C). Moreover, we found that patients with tumors exhibiting high PTTG1 expression exhibited significantly shorter overall survival compared with those having low PTTG1 expression in either the stage I and II subgroup (n = 88; P = 0.001, log-rank; Figure 1D) or the stage III and IV subgroup (n = 58; P = 0.042, log-rank; Figure 1E). Thus, high PTTG1 expression appears to be a risk factor predicting poor survival, suggesting that increased PTTG1 expression likely contributes to NSCLC pathogenesis and might represent a prognostic biomarker for the disease.

PTTG1 promotes NSCLC cells migration

To determine the specific involvement of PTTG1 in the migration of H1299 cells, we applied siRNA technology to inhibit PTTG1 expression. Transfected cells were selected through hygromycin B resistance to establish cell lines that stably downregulated PTTG1 expression. A number of stable clones were obtained using these methods. The siRNAs include PTTG1 siRNA duplex no. 2, which was stably transfected, and similar phenotypes were also obtained (data not shown). Two representative clones (clones 1 and 2) were used in the analysis (Figure 2A). Given that these clones had similar phenotypes, we chose to present the results from clone 1, designated as SiPTTG1/H1299 cells, as the representative. An siRNA construct containing a scrambled sequence was transfected into H1299 cells to generate control cells, which were designated as Scr/H1299 cells. The rate of cell proliferation was examined in both Scr/H1299 and SiPTTG1/H1299 cells in vitro. The doubling time of SiPTTG1/H1299 cells did not differ from that of Scr/H1299 cells (Figure 2B). Chemotaxis assay showed similar results between parental H1299 and Scr/H1299 cells. The SiPTTG1/H1299 cells showed decreased chemotaxis compared with Scr/H1299 cells (Figure 2C).

Meanwhile, PTTG1 stably transfected cell clones were generated through transfection with pcDNA3.1-PTTG1 plasmid and subsequent selection. Thus, we chose to present the results from clone 2, designated as A549/PTTG1 cells, as the representative. A549 cells were also transfected with a pcDNA3.1 vector to establish vector control cells, which were designated as A549/Con. The identification of PTTG1 stably transfected cell clones by western blot analysis is illustrated in Figure 2D. The rate of cell proliferation was also examined in both A549/PTTG1 and A549/Con cells in vitro. The doubling time of A549/PTTG1 cells did not differ from that of A549/Con cells (Figure 2E). The A549/PTTG1 cells showed increased chemotaxis compared with the A549/Con cells (Figure 2F). These chemotaxis results indicate that PTTG1 has an important function in the EGF-induced chemotaxis of NSCLC cells.

Reduction of PTTG1 inhibited EGF-induced F-actin polymerization in NSCLC cells

One of the mechanisms for cell migration toward the leading edge during migration is the polymerization of F-actin toward the cell membrane. The ligand-induced transient F-actin assembly correlates with cellular chemotactic capacity (25,26). To prove that the reduction of PTTG1 could inhibit the EGF-induced chemotaxis of H1299 cells by inhibiting F-actin polymerization, we performed an F-actin polymerization assay. The F-actin fluorescence assay results showed that EGF induced an increase in F-actin content in Scr/H1299 cells, but not in SiPTTG1/H1299 cells. EGF elicited transient actin polymerization at 15 s and 2 min in Scr/H1299 cells, whereas F-actin polymerization was significantly inhibited in SiPTTG1/H1299 cells (Figure 2G), which suggests that PTTG1 has an important function in regulating cytoskeleton rearrangement. F-actin polymerization assay was conducted in A549/Con and A549/PTTG1 cells, and the results are shown in Supplementary Figure S5, available at Carcinogenesis Online.

LIMK and cofilin are two critical factors involved in the regulation of F-actin dynamics; their phosphorylation mediates the regulation of chemotacticants inducing actin polymerization (16). We collected evidence for LIMK and cofilin activation to test whether PTTG1 mediates EGF-induced activation, which consequently regulates actin polymerization. As shown in Figure 2H, a reduction in PTTG1 inhibited EGF-induced phosphorylation of LIMK and cofilin, consistent with the actin polymerization defects in SiPTTG1/H1299 cells.
In summary, our results indicate that PTTG1 regulated EGF-induced NSCLC cell chemotaxis probably through mediating cell actin polymerization.

**PTTG1 promoted NSCLC cell invasion**

Cell migration is a highly dynamic phenomenon essential to morphogenesis, wound healing and cancer metastasis and invasion. We demonstrated that PTTG1 has an important function in H1299 and A549 cell migrations and hence hypothesized that it has a pivotal effect on NSCLC cell invasion. To identify the effects of PTTG1 on NSCLC cell migration and invasion, we used wound healing/scratch assay and matrigel invasion assay. When a scratch was created in the monolayer cells, SiPTTG1/H1299 cells required a longer time to fill the gap compared with the Scr/H1299 cells (Figure 3A). The SiPTTG1/H1299 cells that invaded through the matrigel with 10ng/ml EGF stimulation were considerably fewer compared with the Scr/H1299 cells (Figure 3B, B1). Quantitative analysis of cell numbers revealed that the SiPTTG1/H1299 cells had an invasion rate two times lower than that of Scr/H1299 cells in response to 10ng/ml EGF (Figure 3B, B2). We conducted the same experiment in A549 cells and obtained the same results (Supplementary Figure SB and C, available at Carcinogenesis Online).

---

**Fig. 1.** PTTG1 expression in NSCLC tissues and NSCLC cell lines. (A) A1, Expression of PTTG1 protein in paired NSCLC tissues (T) and ANT tissues, with each paired obtained from the same patient. A2, Expression of PTTG1 protein in cultured human bronchial epithelial cell line (BEAS-2B) and NSCLC cell lines (A549, SPC-A1, Calu1 and H1299). β-Actin was used as a loading control. Quantification of relative protein levels on three different western blots is shown below the blots. The results were from a representative of at least three repeated experiments. (B) Expression of PTTG1 protein in normal lung tissue (a), squamous cell carcinoma with lymph node and distant metastasis (b), squamous cell carcinoma without metastasis (c), adenocarcinoma with lymph node and distant metastasis (d) and adenocarcinoma without metastasis (e) was examined by immunohistochemical staining. Scale bar: 10 µm. (C) Kaplan–Meier analysis of correlation between the PTTG1 level and overall survival of NSCLC patients with high (n = 77) and low (n = 69) PTTG1 expression. (D) Kaplan–Meier analysis of correlation between the PTTG1 level and survival of NSCLC patients in the stage I and II subgroup. (E) Kaplan–Meier analysis of survival of NSCLC patients in the stage III and IV subgroup. P values were calculated by the log-rank test; P < 0.05 was considered statistically significant.
Previous studies have proven that high MMP-2 and MMP-9 expression can promote NSCLC invasion (27). Considering that the different invasive capabilities of PTTG1 reduced NSCLC cells and the control NSCLC cells, we were prompted to detect MMP-2 and MMP-9 expression in Scr/H1299 and SiPTTG1/H1299 cells. As shown in Figure 3C, ELISA showed that the amount of MMP-2 and MMP-9 expression in Scr/H1299 and SiPTTG1/H1299 cells. As shown in Figure 3C, ELISA showed that the amount of MMP-2 and MMP-9 expression in Scr/H1299 and SiPTTG1/H1299 cells.
PTTG1 enhanced NSCLC cells invasion. (A) Quantification of in vitro scratch assays on Scr/H1299 and SiPTTG1/H1299 cells. The distance of cell migration was measured. EGF, 10 ng/ml. *P < 0.05 (two-way ANOVA). (B) B1, representative pictures of Scr/H1299 and SiPTTG1/H1299 penetrated cells. B2, quantification of indicated cells analyzed using the matrigel invasion assay. Scale bars = 50 μm. *P < 0.05 (two-way ANOVA). (C) In the presence of EGF (10 ng/ml), the Scr/H1299 and SiPTTG1/H1299 cells were cultured for 48 h with serum-free medium on a six-well plate. After culture, the culture media were concentrated to assess MMP-2 and MMP-9 secretion using ELISA. *P < 0.05 (two-way ANOVA). (D) Scr/H1299 and SiPTTG1/H1299 cells were treated with EGF (10 ng/ml) for 5 min. After treatment of cells, proteins were extracted and gelatin zymography analysis was performed. (E) Scr/H1299 and SiPTTG1/H1299 cells were treated with EGF (10 ng/ml) for 15, 30 and 90 s. After treatment of cells, proteins were extracted and western blot using antibody to pAkt473 (phosphorylated Akt473), pAkt308 (phosphorylated Akt308) and Akt was performed. Each result is representative from at least three independent experiments. Bars, standard deviation.
MMP-9 was not significantly changed by knockdown of PTTG1 in the absence of EGF. Conversely, when treated with EGF, SiPTTG1/H1299 cells exhibited an evidently lowered amount of MMP-2 and MMP-9 compared with Scr/H1299 cells. Gelatin zymography analysis of the activities of MMP-2 and MMP-9 revealed that both MMP-2 and MMP-9 activities were decreased in the cell lysates of SiPTTG1/
H1299 cells compared with Scr/H1299 cells after treatment with EGF (Figure 3D). The results strongly suggest that the downregulation of PTTG1 is relevant to decreased expressions of MMP-2 and MMP-9 in NSCLC cell lines, indicating that MMP-2 and MMP-9, at least partially, had important functions in the invasiveness of NSCLC cells induced by PTTG1.

The PI3K/Akt pathway is frequently dysregulated in cancer, and the activation of Akt is associated with cell proliferation, survival, migration and invasion (28). The PI3K/Akt pathway is activated in many lung cancer patients (29). Previous studies have shown that PTTG1 promotes the migratory and invasive properties of breast cancer through Akt activation (9). To explore the mechanism by which PTTG1 silencing inhibits the migration/invasion of NSCLC cells, we first observed the activation of the PI3K/Akt pathway. As shown in Figure 4E, EGF-induced phosphorylation of Akt473 and Akt308 was significantly decreased in SiPTTG1/H1299 cells compared with Scr/H1299 cells.

PTTG1 was modulated by miR-186

To associate miRNAs with the regulation of PTTG1 expression, a bioinformatics search was performed for potential miRNAs targeting mRNA of PTTG1 by using two common databases: microRNA.org and TargetScan. The databases predicted miR-186, miR-329 and miR-362-3p as the potential miRNAs targeting PTTG1 (Figure 4A). The three miRNA mimics and NC mimics were synthesized by GenePharma and were transfected into H1299 cells. As shown in Figure 4B, western blot analysis confirmed that the PTTG1 protein levels were indeed reduced drastically in miR-186-transduced cells but pronouncedly elevated in miR-186-silenced cells compared with those in the corresponding control cells. However, miR-186 had no impact on the PTTG1 mRNA levels (Figure 4C). The enhanced miR-329 and miR-362-3p in H1299 cells, however, could not repress PTTG1 protein expression. To identify the effects of miR-186 on NSCLC cell invasion, we employed matrigel invasion assay. The H1299/miR-186 cells that invaded through the matrigel were considerably fewer compared with the H1299/vector cells in response to 10ng/ml EGF. Quantitative analysis of cell numbers revealed that the H1299/miR-186 cells had an invasion rate two times lower than that of H1299/vector cells in response to 10ng/ml EGF (Figure 4D). RNA immunoprecipitation analysis following miR-186 transfection demonstrated that mRNAs of PTTG1 could be specifically recruited to the miRNP complex isolated using anti-Ago1 antibody (Figure 4E). Moreover, we found that transfection of the miR-186 oligonucleotide mimic dose-dependently abrogated the expression of luciferase, and such suppressive effects could be reversed by anti-miR-186 oligonucleotides (Figure 4F). However, mutating four nucleotides in
the seed sequence of the miR-186 oligonucleotide mimic completely abolished their binding to the target 3′-UTRs. A previous study indicated that miR-186 is a tumor-suppressive miRNA in NSCLC and that multiple cell cycle promoters, i.e. cyclin D1, CDK2 and CDK6, are functional targets of miR-186 (15). Taken together, we concluded that miR-186 is capable of directly targeting the 3′-UTRs of the genes of PTTG1.

Reduction of PTTG1 and overexpression of miR-186 decreased NSCLC cell invasion in vivo

The metastatic properties of NSCLC cells were analyzed in vivo through a xenograft transplant model in SCID mice. Tumor cell colonies in mouse lungs were examined through hematoxylin and eosin staining. As shown in Figure 5A–C, the number of tumor nodules decreased in the lungs of mice injected with SiPTTG1/H1299 and H1299/miR-186 mimic cells compared with that in the lungs of mice injected with Scr/H1299 and H1299/vector cells. At the same time, the PTTG1 protein expressions of the tumor xenograft were downregulated in the mice injected with H1299/miR-186 mimic cells (Figure 5D). Thus, our results clearly indicate that PTTG1 can promote metastasis of human NSCLC cells and that miR-186 can modulate PTTG1 in vivo.

Clinical relevance of PTTG1 and miR-186 in human NSCLC

To examine whether the biological effects of PTTG1 and miR-186 on NSCLC invasion are clinically relevant, we examined the miR-186 expression and its correlation with the protein levels of PTTG1 in NSCLC cell lines and clinical specimens of NSCLC paired with non-cancerous para-tumor tissues. Real-time PCR analysis demonstrated that miR-186 was ubiquitously expressed at lower levels in four human lung cancer cell lines compared with cultured human bronchial epithelial cell line BEAS-2B (Figure 6A). As shown in Figure 6B, miR-186 expression was found to be markedly decreased in all 20 collected NSCLC tumor lesions as compared with that in paired ANT lung tissues. These data strongly suggest that miR-186 expression was significantly suppressed in NSCLC. Such an inverse correlation, as Figure 6C shows, was quantitatively confirmed by our western blot assay, which demonstrated that the extent of upregulated PTTG1 expression (denoted by relative gray value) inversely correlated with the degree of miR-186 downregulation, suggesting that the inhibitory effect of miR-186 on the PTTG1 targets is clinically relevant in NSCLC.

Discussion

Our results support the hypothesis that PTTG1 has a key function in NSCLC cell invasion and can be modulated by miR-186. We show that PTTG1 is evidently overexpressed in both NSCLC tissues and NSCLC cell lines. Pathologic investigation of NSCLC patient tissues indicates a significant correlation between PTTG1 expression and clinical staging, as well as Tumor-Node-Metastasis classification of NSCLC. In the detailed survival study, we have shown that high PTTG1 expression is a predictor of poor prognosis for NSCLC patients. In animal experiments, downregulation of PTTG1 through siRNA inhibited the invasive capability of NSCLC cells toward the SCID mice lung, further demonstrating the potential function of PTTG1 in promoting NSCLC invasion. These data imply that PTTG1 detection could provide useful information about treatment strategy and prognosis that cannot be provided by histopathological diagnosis.

Polarized cell migration is a tightly regulated process that occurs during tissue development, chemotaxis and wound healing (16). This process is also highly associated with the infiltration and invasion of tumors (30). In this study, the downregulation of PTTG1 expression by siRNA in the chemotaxis and wound healing assay severely impaired the migration capability of the H1299 cells. Cofilin is an actin-associated protein that belongs to the actin depolymerization factor/cofilin family and is important for actin dynamics regulation (31). LIMK has been reported to phosphorylate cofilin, which consequently regulates actin polymerization (32,33). Our results show
that the downregulation of PTTG1 expression in H1299 cells resulted in attenuated EGF-induced phosphorylation of both LIMK and cofilin, suggesting that PTTG1 directly participates in cytoskeleton rearrangement.

The components involved in the PI3K/AKT signaling pathway are often activated in a wide variety of cancers (17). Therefore, a better understanding of the function of the downstream pathway of PI3K/AKT in cancer will facilitate the development of more potent and selective inhibitors, which should be a useful adjunct to conventional therapies (34). AKT activation was also observed in primary NSCLC tumors, which was reported to be a poor prognostic factor for NSCLC patients with primary tumors or stage I disease (35,36). Previous studies have shown that PTTG1 promotes migratory and invasive breast cancer properties through Akt activation (9). Consistent with previous studies, our results demonstrated that the EGF-induced phosphorylation of both Akt473 and Akt308 was significantly decreased as a response to PTTG1 knockdown, further suggesting that one possible molecular mechanism of PTTG1 participation in NSCLC invasion is the regulation of the PI3K/Akt signaling pathway. MMs, especially MMP-2 and MMP-9, have been proven to have a significant function in facilitating NSCLC invasion (37). Our results show that with the stimulation of EGF, the PTTG1-reduced cells exhibited a down-regulation of MMP-2 and MMP-9 compared with control cells. Thus, we speculate that the reduction of PTTG1 will drastically decrease MMP-2 and MMP-9 expression and consequently decrease invasive capability. A previous study showed that miR-186 expression correlated with patient survival time and that enforced miR-186 overexpression in NSCLC cells inhibited proliferation by inducing G1/S checkpoint arrest. Conversely, RNA-mediated silencing miR-186 expression promoted cell cycle progression and accelerated NSCLC cell proliferation. Cyclin D1 (CCND1), CDK2 and CDK6 were each directly targeted for inhibition by miR-186, and restoring their expression reversed the miR-186-mediated inhibition of cell cycle progression (15). Our results indicate that miR-186 could affect NSCLC invasion. Thus, miR-186, by modulating PTTG1, has an important function on NSCLC invasion.

In summary, considering that NSCLC remains a highly challenging and deadly malignancy and the search for novel therapeutic agents, identifying effective biomarkers for NSCLC diagnosis and prognosis is of great importance. To this end, our present finding shows that the level of PTTG1 expression correlates with clinicopathological features and patient survival in NSCLC and promotes NSCLC cell invasion under miR-186 modulation. Moreover, our findings suggest a novel role of PTTG1 in bridging two major pathways, i.e., LIMK/cofilin and Akt/MMP, for the regulation of NSCLC cell migration and invasion. Thus, PTTG1 and miR-186 may be useful prognostic markers for NSCLC and novel therapeutic targets for NSCLC invasion intervention.

Supplementary material
Supplementary Table 1 and Figure SA can be found at http://carcin.oxfordjournals.org/

Funding
National Scientific Foundation of China (81072068, 81001001 81272319); The Young and Middle-Aged Scientists Research Awards Foundation of Shandong Province (2010BSB14050, BS2011YY060); Foundation of Shandong Educational Committee (J12L03, J13L03).

Conflict of Interest Statement: None declared.

References
PTTG1 modulated by miR-186 regulates NSCLC invasion


Received February 18, 2013; revised April 2, 2013; accepted April 26, 2013