

MicroRNA-187 modulates epithelial-mesenchymal transition by targeting PTRF in non-small cell lung cancer

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Abstract. MicroRNAs (miRNAs) that negatively regulate gene expression play a key role in the development and progression of cancer. Aberrant expression of hsa-miR-187 (miR-187) has been reported in various malignancies. However, the function of miR-187 in tumor progression remains controversial and its role in non-small cell lung cancer (NSCLC) is poorly understood. In the present study, the role of miR-187 in the progression of NSCLC was investigated. Our results revealed that miR-187 was frequently upregulated in NSCLC tissues and cells. Furthermore, ectopic introduction of miR-187 promoted cell migration, whereas miR-187 inhibitor had the contrary effect in NSCLC cells. Of significance, miR-187 induced epithelial-mesenchymal transition (EMT), which plays a pivotal role in the initiation of metastasis and activated mitogen-activated protein kinase (MAPK) and phosphatidylinositol-3-kinase/protein kinase B (PI3K/AKT) pathways. Polymerase I and transcript release factor (PTRF) was identified as a direct target of miR-187 in the promotion of the migration of NSCLC cells. Restored expression of PTRF neutralized the promoting effect of miR-187 on cell migration and EMT of NSCLC cells. Collectively, our data highlight the pivotal role of miR-187 in the progression of NSCLC, indicating this factor as a potential candidate in molecular cancer therapy.

Introduction

Lung cancer remains the leading cause of cancer-related deaths worldwide (1). Non-small cell lung cancer (NSCLC) accounts for ~85% of all lung cancer subtypes and its clinical course is very crucial with poor patient outcomes (2,3). Despite recent advances in chemotherapy and surgical procedures, the overall 5-year survival rate of NSCLC patients (~15%) has not markedly improved (1). Adjacent invasion and distant metastases are responsible for the failure of lung cancer therapy (4). Therefore, further exploration of the underlying mechanisms of NSCLC progression is essential.

Epithelial-mesenchymal transition (EMT) is a key stage in tumor progression. This biological process permits polarized epithelial cells to assume a mesenchymal cell phenotype, however this often induces tumor metastasis (5). Increasing evidence supports EMT as a common event in human advanced NSCLC (6,7). Consequently, to identify new diagnostic strategies and specifically targeted drugs, exploring key molecules in EMT that control NSCLC metastasis is paramount.

MicroRNAs (miRNAs), a family of small non-coding RNAs, are processed from precursor RNAs with a typical hairpin secondary structure (8). Several miRNAs are aberrantly expressed in NSCLC and their dysregulation has resulted in cancer progression and poor clinical outcome (9-11). Considering that miRNAs usually act as EMT-associated downstream effectors of receptor signaling or protein kinases (12), miRNAs may represent novel targets for designing antimetastatic drugs due to their specificity. Moreover, miRNA efficacy data is now available, making miRNA-based technology applicable against NSCLC metastasis *in vivo* (13).

Recently, miR-187 ectopic expression was detected in various types of cancer including nasopharyngeal (14), renal (15), pancreatic (16), prostate (17) and neuroblastoma (18). In NSCLC, miR-187 is also a potential tumor suppressor (19), however the precise molecular mechanism through which miR-187 influences NSCLC progression remains largely unknown. In the present study, we aimed to investigate the

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potential role of miR-187 in NSCLC progression. We also explored the mechanisms underlying tumor metastasis and direct targets of miR-187.

Materials and methods

Cell culture. Human NSCLC cell lines A549, H322, GLC-82, SPC-A1, PC-9, H460 and H1299, and the normal human bronchial epithelial cell line BEAS-2B, were obtained from the Cell Bank of the Chinese Academy of Sciences (Shanghai, China). All cells were authenticated by short tandem repeat (STR) profiling before receipt and were propagated for <6 months after resuscitation. All cells were cultured in RPMI-1640 medium (HyClone, Logan, UT, USA) supplemented with 10% fetal bovine serum (FBS; Gibco-BRL, Invitrogen, Paisley, UK) at room temperature with a humidity of 90-95%, and 5% CO₂.

miRNA transfection. Refer to our previous study for details (20).

Microarray data set analysis. A microarray data set containing 116 paired primary lung cancers, and their corresponding adjacent normal lung tissues (collected a minimum of 5 cm from the tumor) were retrieved from the Gene Expression Omnibus (GEO) database (accession no. GSE15008) (21).

RNA isolation, reverse transcription and quantitative real-time polymerase chain reaction (qRT-PCR). qRT-PCR for miRNAs and target genes was performed as previously described (20,22). Primer sequences used for PCR assays were as follows: U6 forward, 5'-CTCGCTTCGGCAGCAGCACA-3' and reverse, 5'-AACGCTTCACGAATTTGCGT-3'; and miR-187 forward, 5'-TCGTGTCTTGTGTTGCAGCC-3' and reverse, 5'-TGGTGTCTGTTGAGTCG-3'.

Western blot analysis. Protein expression was evaluated by immunoblot analysis of cell lysates (20-60 µg) in RIPA buffer solution using a rabbit antibody to E-cadherin, and mouse antibodies to β-catenin, fibronectin, vimentin and β-actin (1:500; Santa Cruz Biotechnology, Santa Cruz, CA, USA); rabbit antibodies to p-Akt (Ser473), AKT, p44/42 MAPK (ERK1/2), and p-p44/42 MAPK (ERK1/2) [1:1,000; Cell Signaling Technology (CST), Danvers, MA, USA]; and rabbit antibodies to polymerase I and transcript release factor (PTRF) (1:500; Abcam, Cambridge, UK).

Immunofluorescence (IF). Refer to our previous study for details (20). In brief, slides were incubated with a rabbit antibody to E-cadherin, mouse antibodies to β-catenin and vimentin (1:500; Santa Cruz Biotechnology), and rabbit antibody to PTRF (1:100; Abcam) at 4°C overnight followed by washing with phosphate-buffered saline (PBS) 3 times. Coverslips were then incubated with fluorescein isothiocyanate (FITC)- or Texas Red (TR)-conjugated anti-mouse or anti-rabbit secondary antibodies (1:120; Santa Cruz Biotechnology) for 30 min at room temperature prior to staining with 4'-6-diamidino-2-phenylindole (DAPI; Invitrogen, Carlsbad, CA, USA).

Cell migration analysis. Refer to our previous study for details (20).

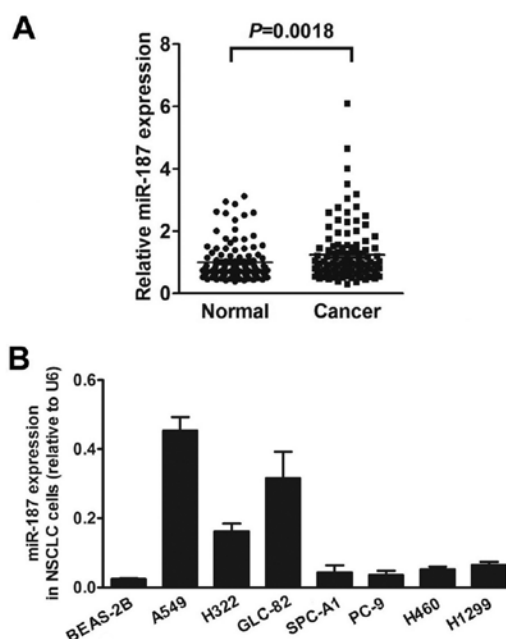


Figure 1. miR-187 is upregulated in NSCLC tissues and cell lines. (A) miR-187 expression in paired human NSCLC and their adjacent normal mucosal tissues from a published high-throughput microarray dataset (NCBI/GEO/GSE15008; n=116). miR-187 expression was normalized to the matched adjacent normal tissues. (B) Increased expression of miR-187 was exhibited in all 7 NSCLC cell lines compared with the normal human bronchial epithelial cell line, BEAS-2B. Error bars represent the mean \pm SD from 3 independent experiments. NSCLC, non-small cell lung cancer.

Wound-healing assay. Refer to our previous study for details (20).

miRNA target validation. The full-length PTRF 3' untranslated regions (3'UTRs) were amplified by PCR and cloned downstream of the firefly luciferase gene in the psiCHECK-2 vector (Promega, Madison, WI, USA). This vector was named the wild-type (wt) 3'UTR. Site-directed mutagenesis of the miR-187 binding site in the PTRF 3'UTR was conducted with the GeneTailor Site-Directed Mutagenesis System (Invitrogen) and named the mutant (mt) 3'UTR. For reporter assays, the wt or mt 3'UTR and miR-187 mimic or inhibitor were cotransfected. The luciferase activity was assessed 2 days after transfection using the Dual-Luciferase Reporter Assay System (Promega). Primer sequences used for PCR assays were as follows: WT PTRF forward, 5'-CCGCTCGAGGAGGCAGCCCCCG CAGTGGACAAC-3' and reverse, 5'-ATAAGAATGCGGCC GCGAAAAGCAGGTTTATTGGTTCGGGC-3'; MutPTRF-1 forward, 5'-TGATTCTGTTTGGACTGGGTTCTCATCTGT GCTCCAGGTTCTCAAGACACGAGTCCCC-3' and reverse 5'-GGGGACTCGTGTCTTGAGAACCTGGAGCACAGAT GAGAACCCAGTCCAAACAGAAATCA-3'; MutPTRF-2 forward, 5'-TTCTCAAGACACGACGAGTTCTCATCTGT GCTGTCCCCTTGTTCCTCCCCATTAAAG-3' and reverse 5'-CTTTAATGGGGAGGAACAAGGGGACAGCACAGAT GAGAACCTGGTTCGTGTCTTGAGAA-3'; and MutPTRF-3 forward, 5'-TTCTCATCTGTGCTCCAGGTTCTCATCTGT GCTGTCCCCTTGTTCCTCCCCATTAAAG-3' and reverse 5'-CTTTAATGGGGAGGAACAAGGGGACAGCACAGAT GAGAACCTGGAGCACAGATGAGAA-3'.

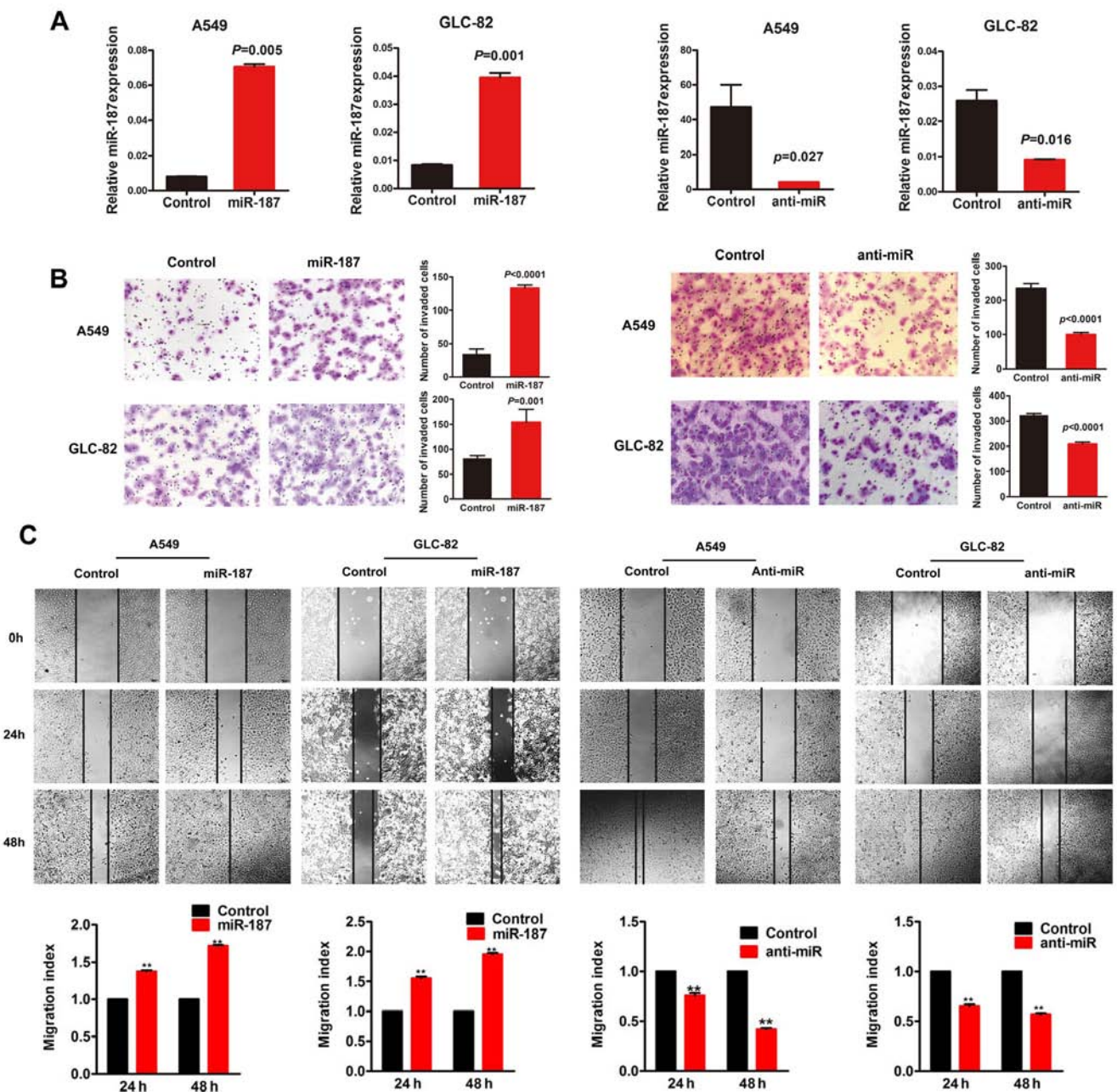


Figure 2. Exogenous miR-187 promotes NSCLC cell migration and motility *in vitro*. (A) RT-PCR analysis of miR-187 expression in miR-187 or anti-miR-187-transduced cells. Transcript levels were normalized to U6 expression. (B) Transwell assay using control and miR-187-overexpressing or -knockdown cell lines. The cells were counted under a microscope in 5 randomly selected fields. The histogram at the right shows the number of invaded cells. (C) Representative images of the wound-healing assay using control and miR-187-overexpressing or -knockdown cell lines. The lower histograms show the migration index of treated or control cells. The distance migrated by the treated cells was relative to that migrated by the control cells. Each bar represents the mean \pm SD. The results were reproduced from 3 independent experiments; ** $P<0.01$. NSCLC, non-small cell lung cancer.

Statistical analysis. Data were analyzed using SPSS software (version 19.0; SPSS, Inc., Chicago, IL, USA). For the qRT-PCR, Transwell and luciferase reporter assays, the Student's t-test or one-way analysis of variance (ANOVA) was carried out. All data are presented as the mean \pm standard deviation (SD). Statistical significance was established when the P-value was <0.05 .

Results

miR-187 is upregulated in NSCLC tissues and cell lines. Microarray analysis showed that miR-187 was upregulated

at an average of 1.26-fold ($P=0.0018$) in the primary lung neoplasms compared with the corresponding adjacent normal lung tissues. Up to a 4.99-fold change in miR-187 expression was found in 71 of all 116 NSCLC samples compared to the controls (Fig. 1A). Furthermore, increased expression of miR-187 was found in all 7 NSCLC cell lines compared with the normal human bronchial epithelial cell line BEAS-2B (Fig. 1B).

Exogenous miR-187 promotes NSCLC cell migration and motility *in vitro*. In the present study, miR-187 mimic oligonucleotides were transfected into A549 and GLC-82

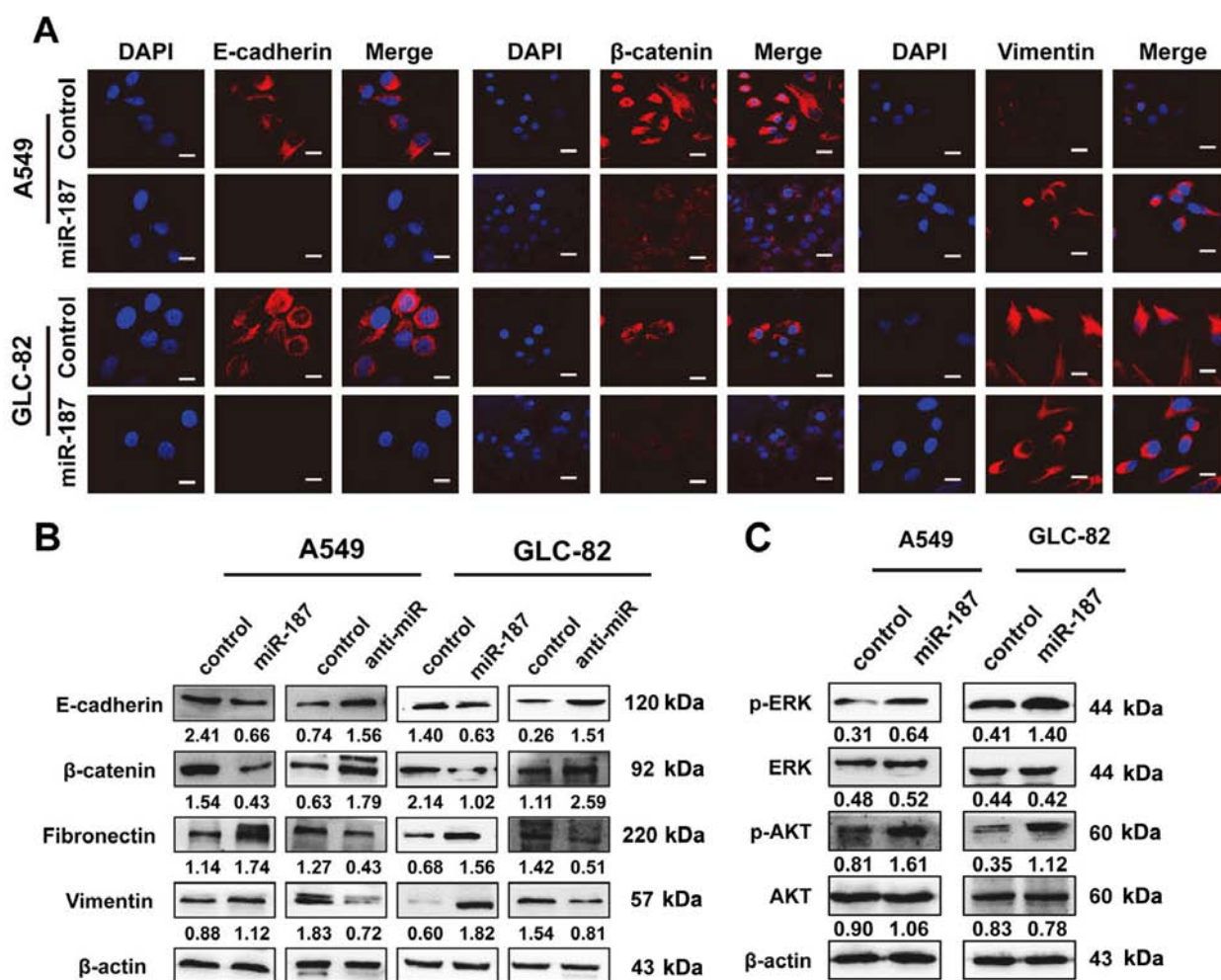


Figure 3. miR-187 mediates EMT and activates signal transduction pathways. (A) IF assays were performed to observe the localization of EMT markers (Scale bar, 10 μ m). (B) Western blot analysis was performed to detect EMT markers. (C) Western blot analysis was performed to detect phosphorylation levels of ERK and AKT. The immunosignal was quantified using densitometric scanning software and the relative protein abundance was determined by normalization with β -actin. Representative figures are shown. The results were reproduced from 3 independent experiments. EMT, epithelial-mesenchymal transition; IF, immunofluorescence.

cell lines to determine their effects on cellular behavior. In contrast, anti-miR-187 was utilized to observe the effect of miR-187-depletion on cellular behavior as an miRNA inhibitor. qRT-PCR was performed to detect the transfection efficiency ($P < 0.05$; Fig. 2A). miR-187-treated cells showed a remarkable increase in migration and motility potential in Transwell and wound-healing assays, whereas depletion of endogenous miR-187 significantly decreased migration and motility ($P < 0.05$; Fig. 2B and C).

miR-187 mediates EMT and activates signal transduction pathways. To investigate the mechanisms underlying miR-187-mediated biological behavior, we investigated its effects on EMT and signal transduction pathways. IF assays revealed that exogenous miR-187 overexpression resulted in decreased expression of the epithelial markers E-cadherin and β -catenin, and increased expression of the mesenchymal marker vimentin (Fig. 3A). Western blot analysis revealed similar changes in EMT markers (Fig. 3B) and indicated that the phosphorylation status of proteins was involved in EMT signaling. As shown in Fig. 3C, miR-187 activated the

mitogen-activated protein kinase (MAPK) pathway through the phosphorylation of p44/42 MAPK (ERK1/2) and phosphatidylinositol 3-kinase/protein kinase B (PI3K/AKT) signaling via the phosphorylation of AKT in A549 and GLC-82 cells.

PTRF is a direct target of miR-187. As indicated by computer-based sequence analysis (based on TargetScan Human 6.2, PicTar and miRanda), PTRF was identified as a potential target of miR-187 and therefore considered to be involved in cell migration. The target sequence (wt 3'UTR) or 2 mutant sequences (mt 3'UTR position 1764-1790) were cloned into a luciferase reporter vector (Fig. 4A), then 293T cells were transfected with the wt or mt 3'UTR vector and an miR-187 mimic. A significant decrease in luciferase activity was observed in both the wt and mt vectors compared with the miR controls, whereas increased activity was found in cells cotransfected with anti-miR-187 and the wt or mt 3'UTR vectors (Fig. 4B). Unlike for double mutations, single mutations in the putative binding sites in the PTRF 3'UTR region could not abrogate this suppression, thereby providing strong evidence of direct interaction between miR-187 and

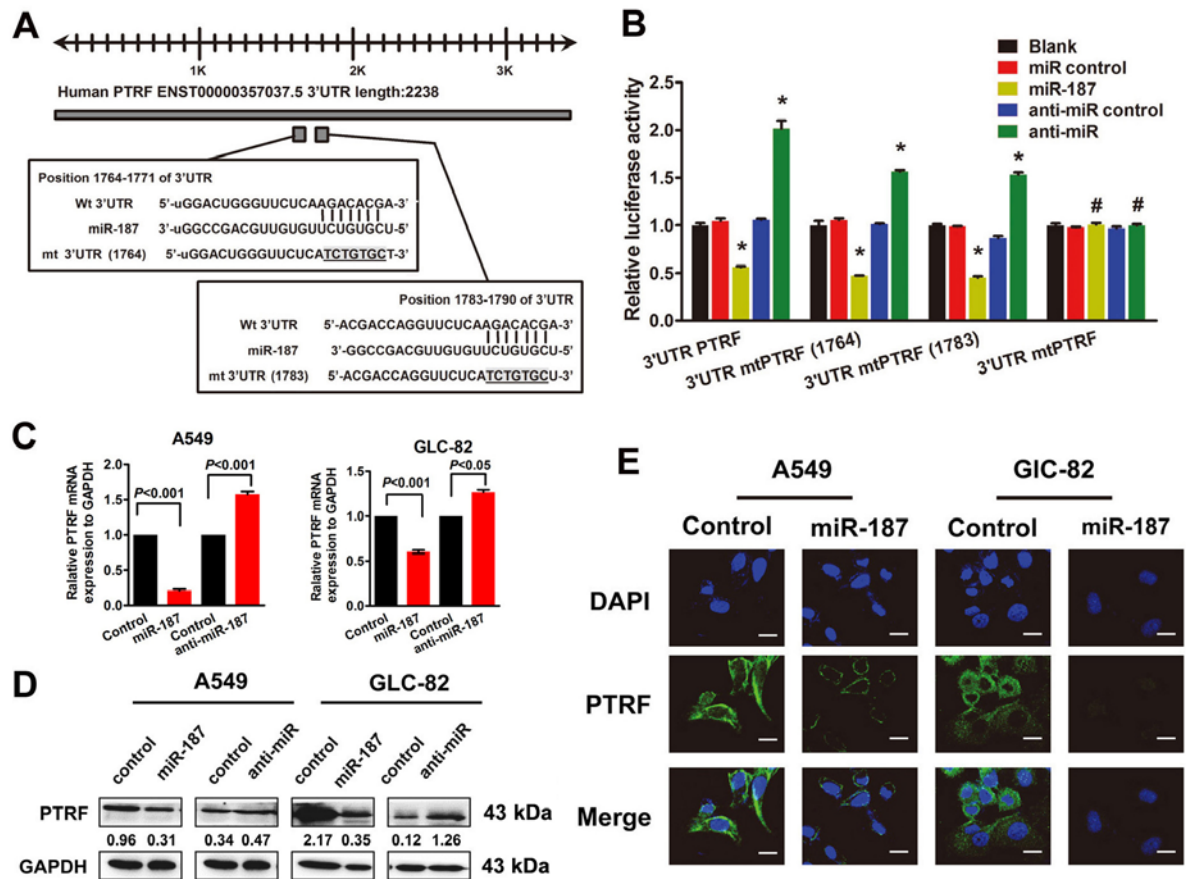


Figure 4. PTRF is a direct target of miR-187. (A) Diagram of PTRF 3'UTR containing 2 putative conserved target sites for miR-187, identified using the TargetScan database. (B) Results of luciferase reporter assays in 293T cells, with cotransfection of wt or mt 3'UTR and miR mimic and inhibitor, as indicated; *P < 0.05 and #P > 0.05. (C) qRT-PCR analysis of PTRF mRNA proteins in indicated cells transfected with miR-187 mimic. Each bar represents the mean \pm SD. (D) Western blot analysis of PTRF protein expression in indicated cells after transfection with miR-187 mimic or inhibitor. Results were reproduced from 3 independent experiments. (E) IF assays were performed to observe localization of the PTRF protein (Scale bar, 10 μ m). PTRF, polymerase I and transcript release factor; IF, immunofluorescence.

PTRF (Fig. 4B). In general, these results strongly suggest that PTRF is a direct target of miR-187 in NSCLC cells.

Additionally, qRT-PCR revealed decreased expression of PTRF in A549 and GLC-82 cells transfected with miR-187 (Fig. 4C). Western blot analysis revealed that transfection of miR-187 led to a markedly decreased expression of PTRF proteins in NSCLC cells (Fig. 4D), which were confirmed by IF assays to be potential target genes regulated by miR-187 (Fig. 4E).

PTRF plays crucial roles in miR-187-induced EMT and migration of NSCLC cells. To further explore the function and role of PTRF in NSCLC, we performed gain-of-function assays. Relatively decreased expression of PTRF was found in H460, GLC-82, SPC-A1 and A549 NSCLC cell lines compared with the normal human bronchial epithelial cell line BEAS-2B (Fig. 5A). Exogenous introduction of PTRF markedly enhanced PTRF protein expression (Fig. 5B) and decreased the capacity of cell migration in A549 and GLC-82 cells (Fig. 5C).

To evaluate the biological role of PTRF in cell migration and EMT induced by miR-187, we rescued PTRF expression in miR-187-transfected NSCLC cells by transfection with PTRF ORF constructs without 3'UTRs. The results

revealed that exogenous introduction of PTRF clearly suppressed miR-187-induced promotion of migratory potential in A549 and GLC-82 cells (Fig. 6A). Consistent with the biological phenotype results, re-expression of PTRF recovered miR-187-induced EMT (Fig. 6B), strongly supporting PTRF as a key mediator of miR-187-induced cell migration.

Discussion

Our present results highlight the importance of upregulated miR-187 expression for NSCLC cells to develop and/or sustain their aggressive phenotype. Aberrant expression of miR-187 has been reported in various types of cancer (14-18). Although changes in expression vary greatly, miR-187 may participate in tumorigenesis in a tissue-specific manner. However, data on the effects of miR-187 in tumor progression are controversial. For example, in one study, antisense-induced suppression of miR-187 caused the inhibition of cell viability in HeLa cells (23), whereas overexpression of miR-187 induced apoptosis and led to decreased proliferation of HeLa cells in another study (24). Hence, enriching our knowledge regarding specific tumors may contribute to better clinical management. In NSCLC, miR-187 was found to be a potential tumor suppressor in tumor development (19), but the precise

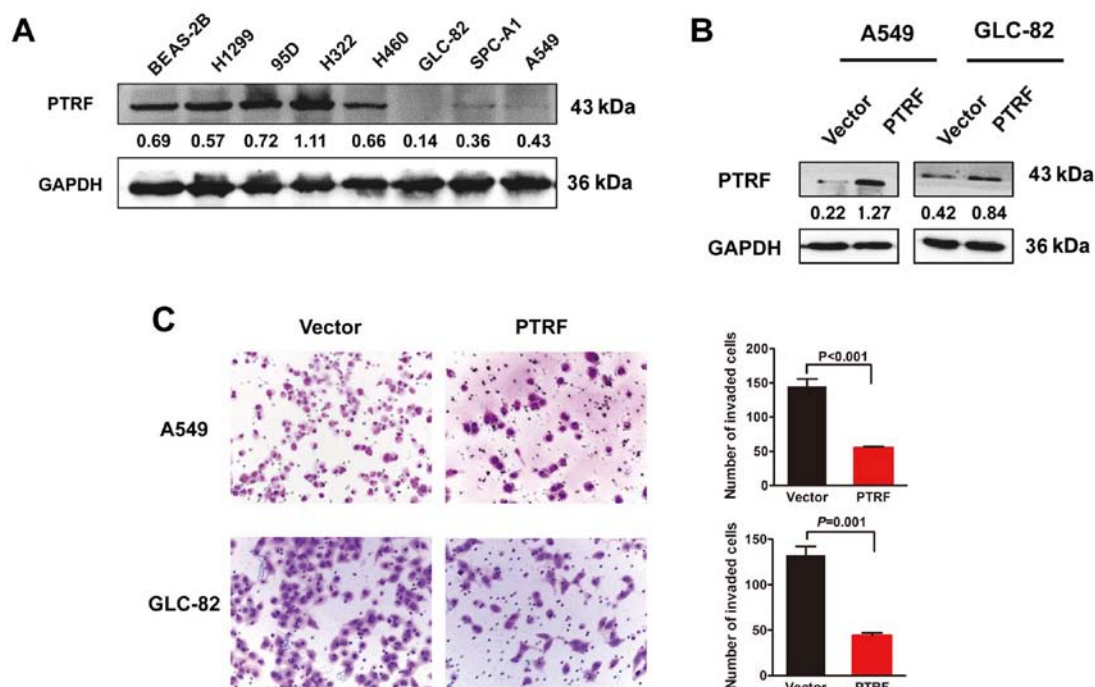


Figure 5. PTRF suppresses migration of NSCLC cells. (A) Expression of PTRF was detected in 7 NSCLC cell lines and in normal human bronchial epithelial cell line BEAS-2B using western blot analysis. (B) Western blot analysis was performed to detect PTRF in indicated cells transfected with the PTRF vector. (C) Transwell assay using control and miR-187-overexpressing or -knockdown cell lines. The cells were counted under a microscope in 5 randomly selected fields. The histograms at the right show the number of invaded cells. Representative figures are shown. The results were reproduced from 3 independent experiments. PTRF, polymerase I and transcript release factor; NSCLC, non-small cell lung cancer.

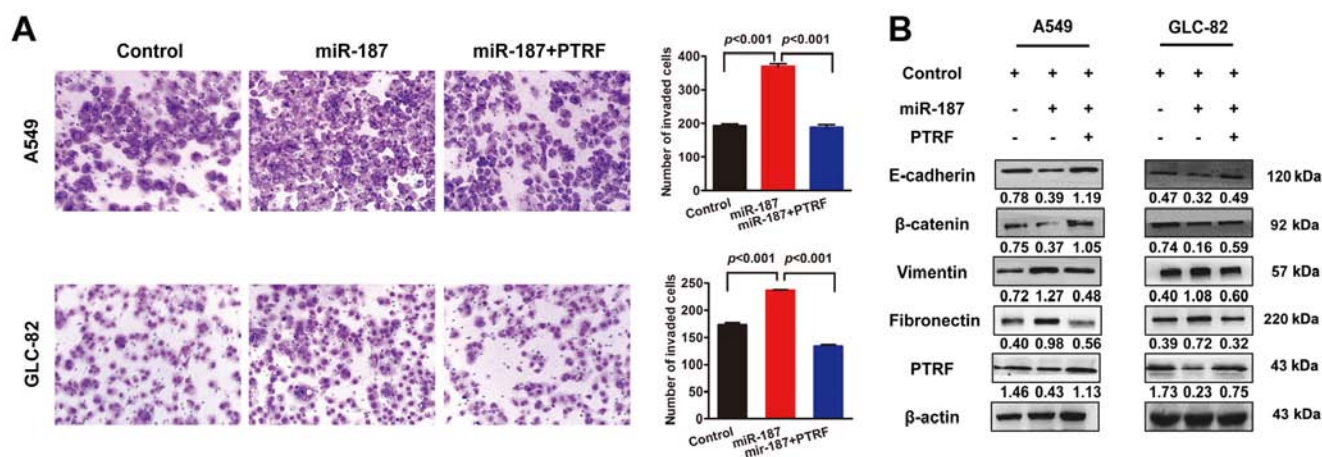


Figure 6. PTRF plays crucial roles in miR-187-induced EMT and migration of NSCLC cells. (A) Transwell assay of indicated miR-187-overexpressing cells co-transfected with PTRF. The cells were counted under a microscope in 5 randomly selected fields. The histograms at the right show the number of invaded cells. (B) Western blot analysis of EMT markers in indicated cells co-transfected with miR-187 and the PTRF vector. Representative figures are shown. The results were reproduced from 3 independent experiments. PTRF, polymerase I and transcript release factor; EMT, epithelial-mesenchymal transition; NSCLC, non-small cell lung cancer.

molecular mechanism through which miR-187 influences NSCLC progression remains largely unknown. In the present study, miR-187 was demonstrated to be a potential promoter of NSCLC progression. However, the mechanism underlying the abnormal expression of miR-187 remains unclear. The present data did not support that abnormal histone or DNA methylation was involved in abnormal expression. Our previous data revealed that miR-187 may be regulated by cell factors in colorectal cancer (CRC), such as transforming growth factor- β

(TGF β). Numerous recent studies have revealed that signaling pathways or non-coding RNAs also regulate miRNA expression (25,26). We may investigate the regulatory mechanism of miR-187 in more detail in future studies.

The mechanism underlying the relationship between miR-187 and tumor aggressiveness remains to be elucidated. EMT is a critical step in tumor progression. Recently, the role of miRNAs in EMT has become a focus in the field of cancer research. Several studies have demonstrated that miR-200

family members act as key regulators of EMT to enforce the epithelial phenotype (27). The suppressive role of miR-187 in ovarian cancer cell lines promoted EMT and was reported to be achieved through targeting of disabled homolog 2 (Dab2) to decrease migration and E-cadherin expression (28). However, these are currently the only observations and suggestions for the underlying molecular mechanisms. In the present study, miR-187 suppressed the epithelial phenotype and induced mesenchymal transition, strongly suggesting its critical role in the EMT process.

TGF β is a known inducer of EMT via Smads and complementary non-Smad pathways, such as MAPK (29-31) and PI3K/AKT (32-34). In our previous study, miR-187 suppressed not only the Smad pathway but also non-Smad pathways in CRC. Restoring expression of miR-187 only partially neutralized TGF β -mediated activation of the Smad pathway by decreasing the phosphorylation level of Smad2 (20). In NSCLC, our data revealed that miR-187 could activate both the MAPK and PI3K/AKT pathways, implying that miR-187 induced NSCLC cell EMT via a non-Smad pathway, not the classical Smad pathway. Therefore, downstream targets must be explored in miR-187-induced EMT, and may be useful in designing novel specific targeted drugs for managing NSCLC metastasis. However, the specific regulator of miR-187 as a metastasis promoter is still unclear.

In general, miRNAs exert their biological function by suppressing their specific target genes at the post-transcriptional level. Recently, B7-H3 (a novel member of the B7 family) and Dab2 were reported as target genes of miR-187 in renal and ovarian cancers, respectively (15,28). SOX4, NT5E and PTK6 expression was increased in CRC cells treated with TGF β , and these effects were offset by the addition of miR-187, suggesting that they are required for TGF β -induced EMT (20). However, we were not able to identify them as targets directly regulated by miR-187 in NSCLC. As previously mentioned, miR-187 may act in a cell- or organ-specific manner through the alteration of the expression of target genes, further changing their phenotypes. In the present study, all the evidence indicates that PTRF may be a direct target of miR-187. Rescued expression of PTRF restored the effect of miR-187 treatment and inactivated the downstream pathway.

PTRF, also known as cavin-1, was originally identified as a protein involved in the dissociation of transcription complexes *in vitro* (35). PTRF in the cell surface membrane is associated with vesicular transport, cholesterol homeostasis (36,37) and lipolysis control (38). To date, the majority of studies related to PTRF have focused on prostate cancer (PC). Changes in the cell membrane involving loss of PTRF expression occur with the development of PC (39). Overexpression of PTRF in PC3 cells decreased cell motility by decreasing matrix metalloproteinase 9 (MMP9) production (40). The absence of PTRF in PC cells was found to contribute significantly to tumor progression and metastasis by promoting the angiogenesis and lymphangiogenesis potential of cancer cells (41). One study supports a role for PTRF/cavin-1, through caveolae formation, as an attenuator of the non-caveolar functionality of Cav1 in Gal3-Cav1 signaling and regulation of focal adhesion dynamics and cancer cell migration (42). In addition, loss of PTRF expression has been demonstrated to be related with tumor progression in breast (43), pancreatic (44) and glioblas-

toma (33) cancers. Only one publication based on proteomic assays reported PTRF/cavin-1 loss-of-expression in NSCLC tissue at the protein level, suggesting a potential role for PTRF in NSCLC development (45). Our results demonstrated for the first time that PTRF contributes to NSCLC progression through its involvement in EMT development.

In short, our results provide a basis for the concept that increased expression of miR-187 in human NSCLC may be significant in the acquisition of an aggressive phenotype. We believe that miR-187 functions as a promoter in NSCLC progression and may serve as a novel therapeutic biomarker. Moreover, the functional and/or mechanistic studies of miR-187 presented in the present study, indicate that miR-187 may play a critical role in controlling non-Smad-mediated EMT by regulating PTRF expression. This suggests that activating EMT during tumor metastasis in NSCLC may be counteracted by suppressing miR-187, a notion that can be readily tested in the clinic.

Acknowledgements

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