Molecules and Cells

MiR-186 Inhibited Migration of NSCLC via Targeting cdc42 and Effecting EMT Process

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In this study, qRT-PCR was employed to identify that miR-186 expression level in NSCLC tissues are highly associated with lymph node metastasis. In addition, through the application of western blotting, luciferase assay and qRT-PCR, it was found that miR-186 targeted 3′UTR of cdc42 mRNA and down-regulated cdc42 protein level in a post-transcriptional manner. Transwell assay indicated that cdc42 partially reversed the effect of miR-186 mimics. Besides, miR-186 was proved to regulate EMT by influencing biomarkers of this process and cell adhesion ability. Thus, miR-186 is a potential target for NSCLC therapy. miR-186 is proposed to be one of tumor-suppressors and may serve as a therapeutic target in NSCLC treatment.

Keywords: cdc42, EMT, migration, MiR-186, NSCLC

INTRODUCTION

Incidence rate of lung cancer ranks in the top 2 among various types of cancers. With almost 1.4 million deaths a year, lung cancer is also the most common reason of cancer-related mortality throughout the world (Hornberger et al., 2015). Non-small cell lung cancer (NSCLC) accounts for approximately 80% of all lung cancer cases, such as squamous cell carcinoma, adenocarcinoma and large cell carcinoma (de Marinis and Grossi, 2008; Jemal et al., 2010). Recently, more new techniques have been employed to allow earlier diagnosis and more effective treatment. However, studying mechanism of NSCLC metastasis still needs to be clarified in details, which is urgent and will contribute to a better prognosis.

MicroRNA (miRNA) is a type of non-coding RNA with 21-25 nucleotides, which regulates target genes at post-transcriptional level (Yu et al., 2012). Almost 30% of protein-coding genes can be regulated by miRNAs which have been shown to play a vital role in biological function (Profumo and Gandellini, 2013). A number of studies indicated that miRNAs can regulate the metastasis process as both positive and negative roles (Ji et al., 2017). In previous studies, miR-186 has been identified to be able to regulate epithelial and mesenchymal transitions during idiopathic pulmonary fibrosis (Lei et al., 2016). More researches revealed that miR186 exotic expression is associated with VEGF modulation in prostate cancer (Terzuoli et al., 2016) and promotes inflammatory cytokines secretion in macrophage (Yao et al., 2016). Nevertheless, the exact mechanism beneath miR-186 affecting NSCLC metastasis still needs to be elucidated.

In this study, exotic expression of miR-186 was validate in NSCLC and its effect on inhibiting NSCLC migration was exhibited by targeting 3′UTR of cell division cycle 42 (cdc42) mRNA. By affecting filopodia localization specifically, Cdc42 plays an important role in regulating cell polarity and migra-

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tion (Ridley, 2015). Additionally, Cdc42 belongs to Rho-GTPase families involving in various signaling pathways, which results in a complicated regulation network that needs to be clarified urgently.

MATERIALS AND METHODS

Cell culture and patient samples

Non-small cell lung cancer cells A549, NCI-H1650 and PC-9 were purchased from ATCC (USA). A549, NCI-H1650 and PC-9 cells were cultured in RPMI 1640 medium while HEK-293T cell was cultured in DMEM with 10% fetal bovine serum (FBS, Gibco, USA) in condition of 5% CO_2 and 37℃. NSCLC and adjacent normal lung tissues were obtained from 50 patients who were diagnosed as NSCLC and treated at The Tumor Hospital of Jilin Province. Clinical researches has been approved by ethics committees.

Plasmid construction

We cloned 3'UTR of cdc42 harboring miR-186 predicted sites to luciferease reporter vector (pmiR-Report, Ambion, USA) for luciferease assays, and to pcDNA3.1 vector for up-regulating cdc42. we named luciferease recombinant plasmid as pmiR-cdc42 and over-expression plasmid as pcD-cdc42. Two siRNA sequences for AGO2 were designed and systemized by Biomics, China. Primer sequences and AGO2 siRNA sequences were list below. Primer sequences were list below.

Primers for luciferease assay:
Forward: 5′ TCAAGTTAGGAGGATGGAGG 3′
Reverse: 5′ TTTTTTTGAAGGGTTTTTGT 3′

Primers for over-expression plasmid:
Forward: 5′ ACTTCCGCGGGCACCCAACT 3′
Reverse: 5′ GAAGGGTTTTTGTTTGTTTAATACATCTGA 3′

SiRNA of AGO2
SiRNA-1:
GCACGGAAGUCCAUCUGAAUU
UUCAGAUGGACUUCCGUGCUU

SiRNA-2:
GCAGGACAAAGAUGUAUUAUU
UAAUACAUCUUUGUCCUGCUU

Transfection

Cells used for transfection must grow at least 50% confluence, and then transfected with miR-186 mimics, miR-186 inhibitor, recombinant plasmid, siRNA for cdc42 and related negative controls with Lipofectamine 2000 (Invitrogen, USA) according to manufacturer's protocol. All of miRNA mimics, miRNA inhibitor, siRNA and negative controls were diluted into 50 nM for use and 0.5 μg recombinant plasmid was transfected in 24-well plate.

Total RNA extraction and real-time PCR analysis

Total RNA in A549, NCI-H1650 and PC-9 cells was extracted with Trizol reagent (Invitrogen, USA). The first strand of cDNA was synthesized using M-MLV (Promega, USA) according to protocol. Real-time PCR (qPCR) were performed on ABI StepOnePlus Real-Time PCR System (Applied Biosystems, USA) and EzOmics SYBR qPCR kit (Biomics, China) was adopted for detecting. The expression level of miR-186 was normalized to U6 snRNA and relative expression level of miR-186 in each group was measured through the $2^{-\Delta\Delta ct}$ method. Primers used for cdc42 detecting were listed below.

Forward: 5′ AGGTGTGTGCTGCTATGAAC 3′
Reverse: 5′ CAGGGCATTTGTCATTATTG 3′

Transwell invasion assay

As for invasion assay, 1×10^5 A549, NCI-H1650 or PC-9 cells were suspended in 200 μl serum free RIPM 1640 medium and then plated cells onto BD BioCoat Matrigel Invasion Chambers (a pore size of 8 μm; BD Biosciences). The lower chamber was filled with RIPM 1640 medium containing 10% FBS. After being cultured 48 h, invasion cells were stained by violet crystalline solution according to protocol. In order to count cell numbers in five random fields, phase contrast microscopy was employed.

Western blotting

Total proteins in cells were extracted and proteins were quantified using BCA method. Through the application of 10% SDS-polyacrylamide gel, 30 μg of proteins were separated and transferred onto polyvinylidinedifluoride membranes. Membranes were blocked with 10% non-fat dried milk dissolved in TBS containing 0.5% Tween 20 for 1.5 h and then incubated with anti-cdc42 RabMAb (1:2000, Abcam, USA), anti-E-cadherin (1:1000, Abcam, USA), anti-vimentin (1:1000, Abcam, USA), anti-α-SMA (1:1000, Abcam, USA) over night at 4℃, during which anti-human β-actin monoclonal antibody (1:500, ZSGB-bio, China) was considered as the control. Moreover, secondary antibody was used according to protocol and developed with ECL system (Thermo Fisher, USA).

Luciferease assay

HEK-293T cells were co-transfected with miR-186 mimics and pmiR-cdc42 -WT/MUT. Cells were lysed and luciferase activity was determined after being cultured for 48 h according to protocol. Containing expression cassette, β-gal was adopted to normalize fluorescent values expressed from pmiR-Report constructs.

Cell adhesion assay

Briefly, Matrigel basement was dissolved at 4℃ overnight. Matrigel was diluted by RIPM 1640 medium with ratio of 1:100. 200 μl diluted matrigel was added into 96-well plate and embedded 4 h at 37℃. after that, A549, NCI-H1650 and PC-9 cells were seeded into 96-well plate. The number of each cell is 10^5 per well. Cells were incubated in 37℃ for 60 min and then washed non-adherent cells by PBS. Microscopy was used for counting adherent cell number after stained by violet crystalline.

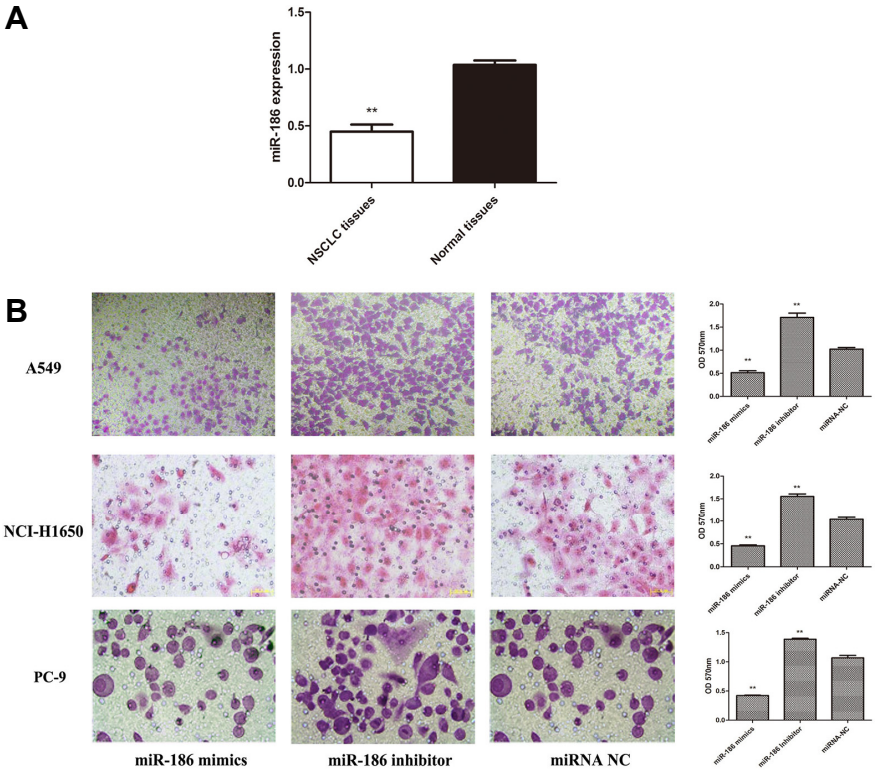

Fig. 1. Expression level of miR-186 in non-small lung cancer tissues and adjacent normal lung tissues. (A) qRT-PCR was employed to measure miR-186 in tissues (**$p < 0.01$). MiR-186 reduced invasion ability of NSCLC cells. (B) Transwell assay was used to determine the effect of miR-186 on NSCLC. MiR-186 mimics reduced invasion ability and miR-186 inhibitor induced it in A549, NCI-H1650 and PC-9 when compared to negative control group (**$p < 0.01$).

Statistical analysis

Statistical testing was conducted with SPSS 17.0 software and Graphpad 5.0 software. Data was analyzed by student's *t* test and all data was presented as Mean ± Standard deviation (SD). Results were considered significant when *$p < 0.05$ and **$p < 0.01$.

RESULTS

Down-regulation of miR-186 in NSCLC

qRT-PCR was employed to clarify miR-186 level in 50 NSCLC samples provided by The Tumor Hospital of Jilin Province and the results were presented in Supplementary data 1. As qRT-PCR results showed, miR-186 expression level in NSCLC tissues was found to be negatively associated with lymph node metastasis ($p < 0.05$). However, no significant differences of miR-186 level were observed in tissues from different gender, age or tumor differentiation. Besides, qRT-PCR results (Fig. 1A) indicated a much lower miR-186 level in NSCLC tissues when compared to adjacent normal tissues ($p < 0.05$).

Lung cancer cells invasion is inhibited by miR-186 up-regulation

Trans-well migration assay was performed to measure invasive activity of A549, NCI-H1650 and PC-9 cells when transfected with miR-186 mimics, miR-186 inhibitor and negative control (NC). In Fig. 1B, it was observed that the number of A549, NCI-H1650 and PC-9 cells penetrating from upper membrane were not significantly different in NC group. However, the penetrating ability of these three cell lines were much higher than those in NC groups when down-regulating miR-186 level, which implied that lung cancer cells invasion was reduced by miR-186 up-regulation.

cdc42 is a direct target of miR-186

Previous studies found that miRNA can regulate cell activity by affecting mRNA level (Chou et al., 2016). In this paper, online prediction software was adopted to predict targets of miR-186. As shown in Fig. 2A, there are two predicted seed regions on 3'UTR of cdc42 for miR-186. In order to verify if cdc42 is a direct target of miR-186, luciferase assay with recombinant pmiR-Reporter plasmids harboring wild type or mutant type seed region sequence of cdc42 was performed for miR-186. Luciferase assay results showed that when compared to NC group, luciferase activity of wild type 3'UTR recombinant plasmid was significantly reduced when co-tranfected with miR-186 mimics (Fig. 2B). Transfection efficacy was confirmed by qRT-PCR (Fig. 2C). Furthermore, subsequent western blotting (Fig. 2D) confirmed that when compared to NC group, miR-186 mimics decreased while miR-186 inhibitor increased cdc42 protein level in A549, NCI-H1650 and PC-9 cells. Nevertheless, miR-186 cannot affect cdc42 mRNA level measured by qRT-PCR (Fig. 2E). Meanwhile, when we knocked down AGO2 protein level in cells by two siRNAs (Fig. 2F), it can be found that miR-186 mimics lost its effect on reducing cdc42 protein level (Fig. 2G). All data above suggested that cdc42 is a direct target of miR-186 and can be regulated in post-transcriptional level.

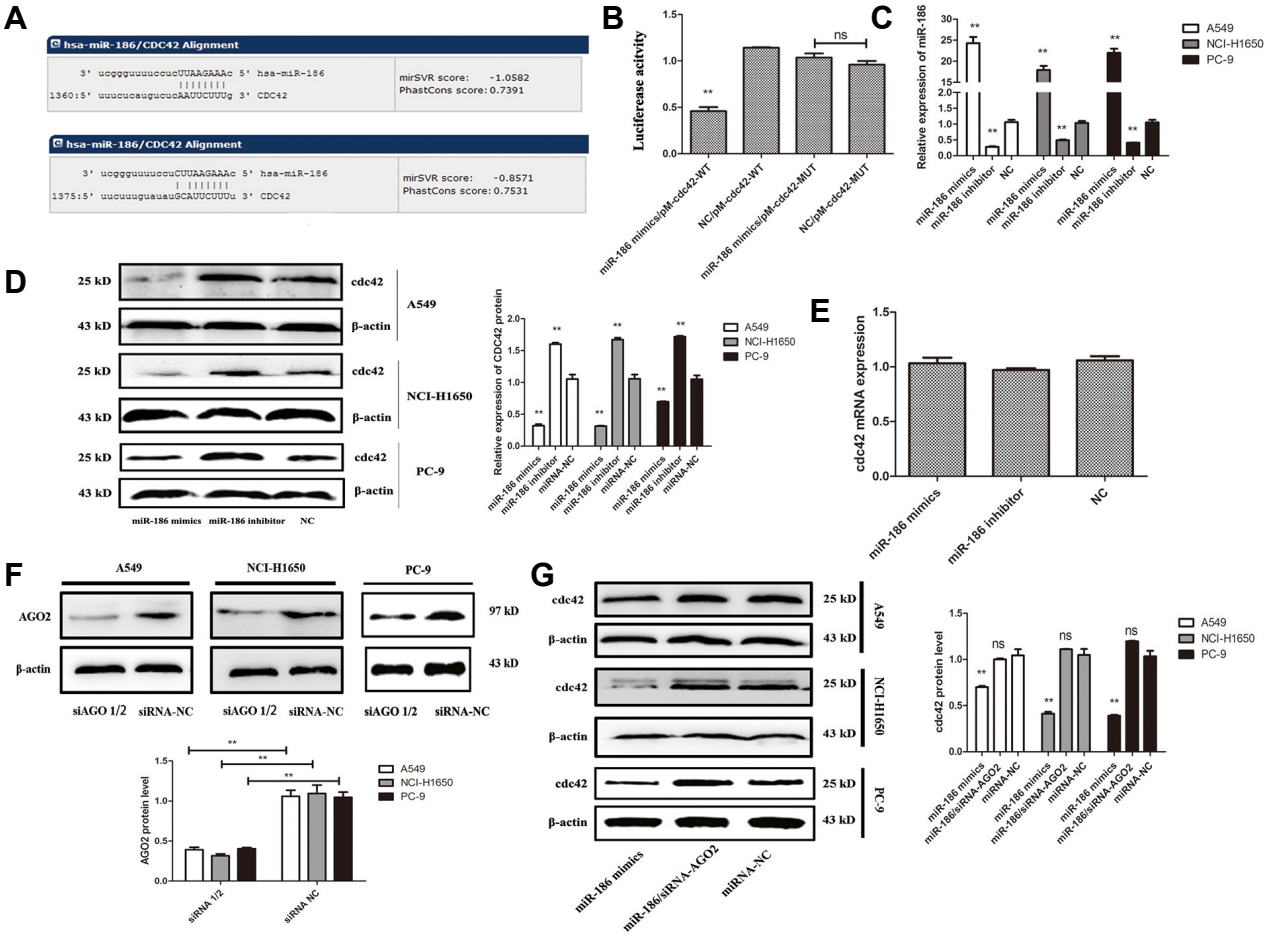

Fig. 2. Cdc42 is the target of miR-186. (A) There are two predicted binding sites of miR-186 on cdc42 mRNA 3'UTR. (B) Luciferease assay was employed to identify directly binding between miR-186 and cdc42 mRNA 3'UTR (**$p < 0.01$). (C) qRT-PCR was used to ensure transfection efficacy of miR-186 mimics, inhibitor compared to NC group in A549, NCI-H1650 and PC-9 cells (**$p < 0.01$). (D) miR-186 mimics down-regulated cdc42 protein while miR-186 inhibitor up-regulated it tested by western blotting in A549, NCI-H1650 and PC-9 cells (**$p < 0.01$). (E) miR-186 cannot effect level of cdc42 mRNA measured by qRT-PCR. (F) Two siRNAs were designed to knock down AGO2 protein in A549, NCI-H1650 and PC-9 cells. (G) miR-186 mimics lost its function of reducing cdc42 protein when co-transfected with siRNA of AGO2 (**$p < 0.01$).

Taken it together, miR-186 inhibits cdc42 transcription and negatively regulates cdc42 protein expression by directly binding to seed sequence on 3'UTR of cdc42.

cdc42 up-regulation partially reverses the effect of miR-186 mimics

Cdc42 recombinant plasmid was designed and transfected into A549, NCI-H1650 and PC-9 cells. The results of western blotting in Fig. 3A revealed that recombinant plasmid significantly induced cdc42 protein expression when compared to empty plasmid group. As compared with miR-186 mimics group, invasive ability of A549, NCI-H1650 and PC-9 cells was reversed when co-transfected with miR-186 mimics and cdc42 recombinant plasmid in trans-well assay (Fig. 3B). According to these results, it is concluded that miR-186 induces invasive ability of A549, NCI-H1650 and PC-9 cells by means of regulating cdc42.

miR-186 affects migration of lung cancer cells related to EMT process

Epithelial-Mesenchymal Transition (EMT) biomarkers were tested when up- or down-regulated miR-186 level in A549, NCI-H1650 and PC-9 cells. It was found that epithelial biomarker E-cadherin increased after miR-186 up-regulation, while mesenchymal biomarkers vimentin and α-SMA decreased. On the contrary, E-cadherin decreased, while vimentin and α-SMA increased when miR-186 level was down-regulated by applying western blotting (Figs. 4A-4D). Due to the results showed that vimentin level can be down-regulated by miR-186 mimics strikingly, we measured if this effect depends on cdc42. It can be identified that cdc42 partially reversed level of vimentin compared to miR-186 mimics group in A549, NCI-H1650 and PC-9 cell (Figs. 4E and 4F). To further support our points, adhesion assay was conducted to verify cell adhesion ability after treated with

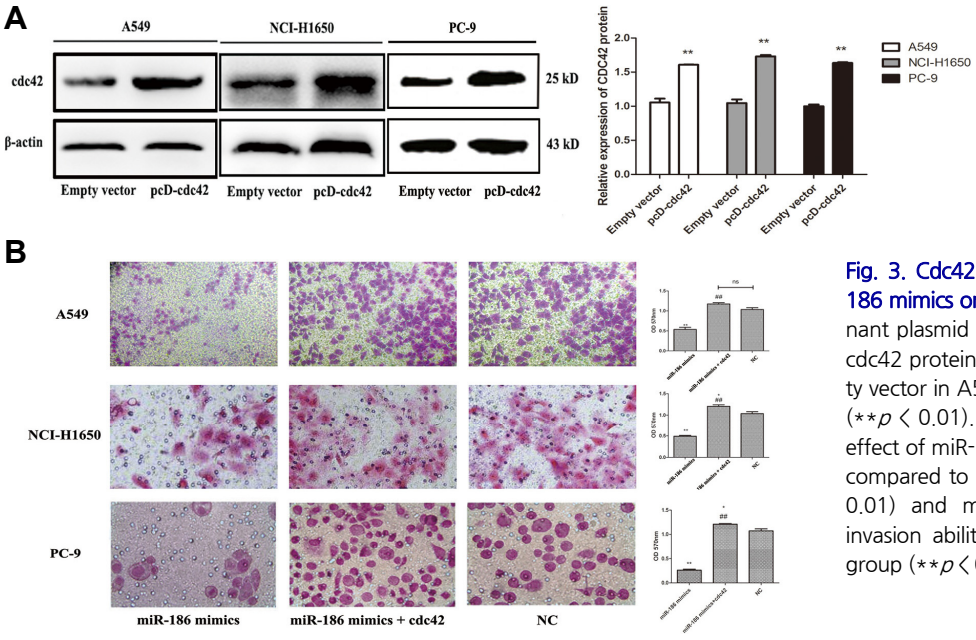

Fig. 3. Cdc42 partly reversed effect of miR-186 mimics on NSCLC invasion. (A) recombinant plasmid of cdc42 (pcD-cdc42) induced cdc42 protein expression compared to empty vector in A549, NCI-H1650 and PC-9 cells ($**p < 0.01$). (B) pcD-cdc42 partly reversed effect of miR-186 mimics on NSCLC invasion compared to miR-186 mimics group ($##p < 0.01$) and miR-186 mimics also reduced invasion ability of NSCLC compared to NC group ($**p < 0.01$).

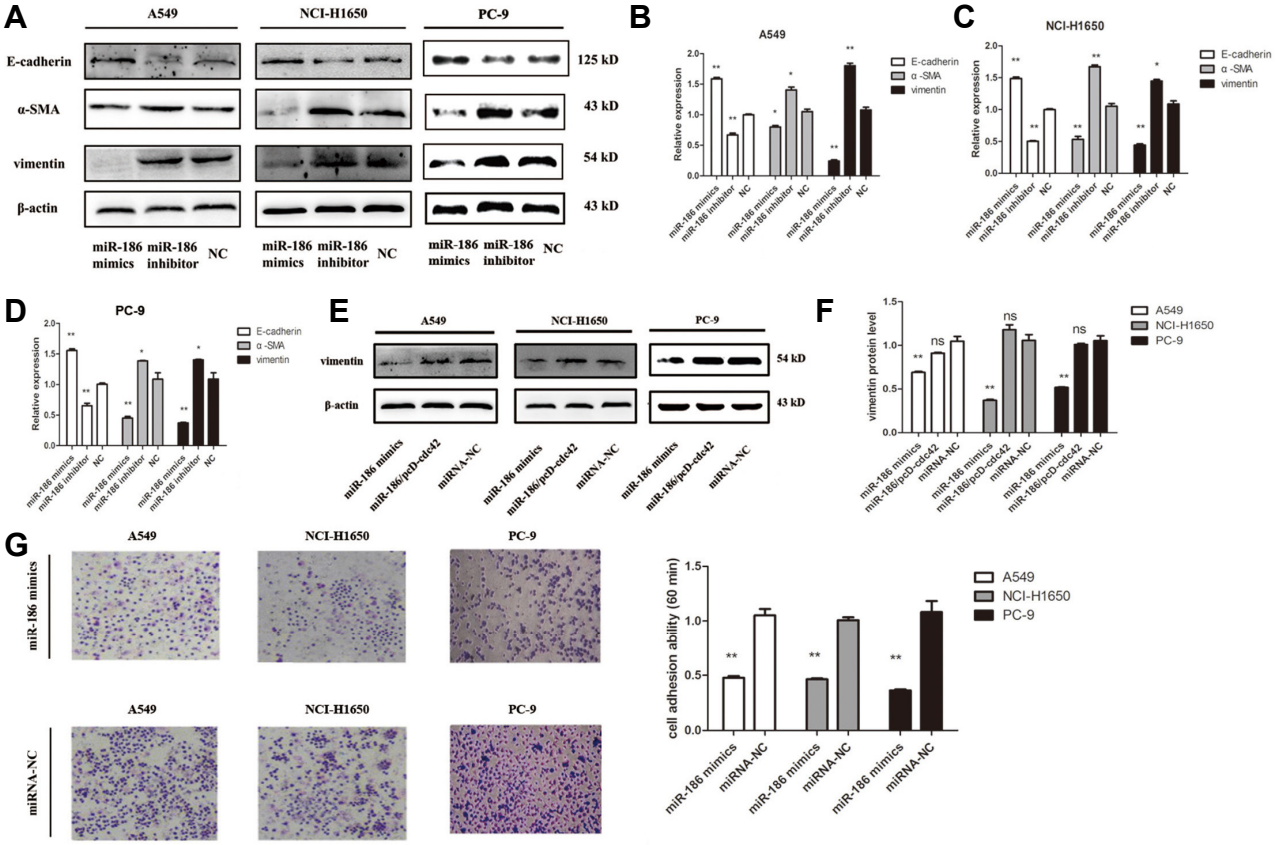

Fig. 4. MiR-186 inhibited invasion of NSCLC by effecting EMT. (A) miR-186 mimics down-regulated α-SMA and vimentin while up-regulated E-cadherin compared to NC group in A549, NCI-H1650 and PC-9 cells verified by Western blotting. (B, C, and D) The results calculated by Quantity One software ($*p < 0.05$, $** p < 0.01$). (E, F) Vimentin is regulated by miR-186 mimics and this process is cdc42 dependent. Cdc42 can partially reverse miR-186 mimics effect on vimentin ($** p < 0.01$). (G) miR-186 mimics reduced adhesion ability of A549, NCI-H1650 and PC-9 cells when compared with NC group ($** p < 0.01$).

miR-186 mimics. In Fig. 4G, it is obvious that all of three NSCLC cell lines had a reduced adhesion ability after transfected with miR-186 mimics for 60 min. These results indicated that miR-186 reduces cells invasion by regulating EMT process especially adhesion ability.

DISCUSSION

As a type of non-coding RNA with 21-25 nucleotides, miRNA regulates target genes in a post-transcriptional and sequence-specific manner, which has been identified to function as tumor suppressors or oncogenes in multiple biological processes, including cell proliferation, oncogenesis, angiogenesis, invasion and migration (Jikuzono et al., 2013; Profumo and Gandellini, 2013; Sun et al., 2013). Previous studies showed that miR-186 was down-regulated in a number of diseases covering cancer. MiR-186 has been found to regulate collagen V and EMT process during idiopathic pulmonary fibrosis and modulate VEGF in prostate cancer (Terzuoli et al., 2016). Besides, researchers proved that miR-186 inhibited aerobic glycolysis in gastric cancer by regulating HIF-1a (Liu et al., 2016a) or inhibited cell proliferation in multiple myeloma (Liu et al., 2016b). Moreover, miR-186 played a significant role in macrophage (Yao et al., 2016) or Alzheimer's disease (Ben Halima et al., 2016). These findings imply us to uncover the role of miR-186 in NSCLC.

In present study, miR-186 was found to be down-regulated in human NSCLC tissues for the first time when compared to adjacent normal tissues. It was demonstrated that ectopic expression of miR-186 by miRNA mimics significantly inhibited invasion ability of A549, NCI-H1650 and PC-9 cells when compared to miRNA NC group, while miR-186 inhibitor induced cell invasion, which suggested that miR-186 down-regulation correlated with clinical NSCLC metastasis and functioned as a tumor-suppressing miRNA. Online software Targetscan 2.0 and miRNA22 were employed to indicate that cdc42 is a target of miR-186 and it is likely that miR-186 reduces the metastasis ability of NSCLC by binding with cdc42.

cdc42 plays an important role in regulating cell polarity and migration by specifically affecting filopodia localization (Ridley, 2015). Cdc42 belongs to Rho-GTPase families involving in various signaling pathways, which plays key roles in establishing cell migratory polarity and migratory persistence. Cdc42 is active in lamellipodial regions and Rac/cdc42-activatied PAK family promotes adhesion turnover based on integrin (Bahri et al., 2010). In addition, 3D modules in vivo showed that cdc42 was a special and key molecule in fibroblast migration process (Zatulovskiy et al., 2014). Cdc42 is guaranteed to be recruited by leader cells and determines moving direction (Etienne-Manneville, 2004). In the current study, it was identified that miR-186 targeted 3'UTR of cdc42 mRNA and down-regulated cdc42 protein level in a post-transcriptional manner by employing western blotting, luciferase assay and qRT-PCR. Transwell assay indicated that cdc42 partly reversed the effect of miR-186 mimics. Taken together, our findings suggest that miR-186 may act as a tumor suppressor by targeting cdc42. Besides, miR-186 is proved to regulate EMT by influencing biomarkers of this process, such as E-cadherin, vimentin and a-SMA and having impact on cell adhesion ability, which is cdc42 dependent and exhibits more directions to conduct following studies. However, prediction software does not show that there are binding sites on vimentin and a-SMA of miR-186 mimics, thus it may exist mediate factors connecting EMT biomarkers changes and miR-186-cdc42 regulatory network. In addition, as there are two main binding sites were predicted, it also needs to clarify if these two binding sites have an equal function in this regulatory work. More structure studies that can make RNA secondary structure clear are needed in future research.

In conclusion, it is identified that miR186 is down-regulated in NSCLC tissues and miR-186 reduces invasion ability of A549, NCI-H1650 and PC-9 cells by directly targeting cdc42 and modulating EMT process which effects on cell adhesion. According to these findings, miR-186 is proposed to be one of tumor-suppressors and may serve as a therapeutic target in NSCLC treatment.

Note: Supplementary information is available on the Molecules and Cells website (www.molcells.org).

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