miR-200b/c targets the expression of RhoE and inhibits the proliferation and invasion of non-small cell lung cancer cells

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Abstract. Lung cancer is a major cause of mortality worldwide and non-small cell lung cancer (NSCLC) accounts for ~80% of all cases of lung cancer. Increasing evidence indicates that Rho family GTPase 3 (RhoE) is important in the carcinogenesis and progression of NSCLC. In addition, several studies have indicated that microRNA (miR)-200b/c is downregulated in NSCLC cells. However, the exact mechanism remains to be elucidated. In the present study, immunohistochemistry (IHC) assays were used to analyze the RhoE and epithelial-mesenchymal transition (EMT)-related proteins in NSCLC tissues. Putative target sequences of the RhoE 3' untranslated region (3'UTR) for miR-200b/c were detected using bioinformatics analysis. The mRNA expression levels of RhoE and miR-200b/c were determined by reverse transcription-quantitative polymerase chain reaction (RT-qPCR) analysis, and western blot analysis was used to detect the protein levels of RhoE in cells. The luciferase-reporter activity of the RhoE 3'UTR was detected using a dual-luciferase assay. A cell counting kit-8 assay, flow cytometry and Transwell assay were used to detect cell proliferation, cell cycle, and invasion and migration ability, respectively. The IHC assays indicated that RhoE was overexpressed in NSCLC tissues. The bioinformatics analysis revealed that the RhoE 3'UTR contained a putative target site for miR-200b/c, which was conserved across species. The results of RT-qPCR analysis showed that the mRNA expression of RhoE was overexpressed and miR-200b/200c was decreased in lung cancer tissues.

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Abbreviations: RhoE, Rho family GTPase 3; ERK1/2, extracellular signal-regulated kinase 1/2; miRNA, microRNA; Si-RhoE, small interfering RNA targeting RhoE-coding sequences

The enhanced expression of miR-200b or miR-200c significantly downregulated the expression of RhoE at the mRNA and protein levels in A549 and NCI-H1299 NSCLC cells. Furthermore, luciferase assays showed that miR-200b and miR-200c directly targeted the 3'UTR of RhoE. The forced expression of miR-200b or miR-200c markedly inhibited A549 cell and NCI-H1299 cell proliferation, G0/G1 progression and cell invasion, which was consistent with the effects of RNA interference-mediated RhoE knockdown in these cells. The suppression of RhoE regulated the expression of EMT-related markers, which was consistent with the effect of miR-200b/c in NSCLC cells, and the expression of EMT-related proteins and RhoE were also correlated in the lung cancer tissues. Therefore, miR-200b and miR-200c targeted the expression of RhoE and inhibited the malignancy of NSCLC cells, and the downregulation of miR-200b and miR-200c may contribute to the high expression of RhoE in NSCLC.

Introduction

Lung carcinoma has the highest cancer-associated mortality and incident rates in the developed world (1). There were 1,800,800 new diagnosed cases of lung cancer (12.8% in total), with 1,600,000 individuals succumbing to mortality in 2012. The number of newly diagnosed lung cancer increased to 14%, accounting for 1/4 cases of cancer-associated mortality by 2016 (1). The annual increase of lung cancer cases is almost 1,300,000, and ~80-85% of these are diagnosed as non-small cell lung cancer (NSCLC), including lung adenocarcinoma, squamous cell lung carcinoma and large cell lung carcinoma (2,3). Molecular target therapy is widely used in the treatment of lung cancer, including epidermal growth factor receptor tyrosine kinase inhibitors and anaplastic lymphoma kinase fusion gene inhibitors and other drugs, which can effectively improve survival rate in specific individual patients with lung cancer at present. However, the five-year survival rate of NSCLC remains <20% (4). The development of lung cancer treatment is limited due to drug resistance and the recurrence of malignancy.

Previous studies investigating the mechanism of the development of lung cancer have focused mainly on the Janus kinase-signal transducer and activator of transcription, mammalian target of rapamycin, and Wnt signaling pathways. MicroRNA (miRNA or miR), which is ~19-25 bp

in length, is a class of endogenous non-encoding RNAs with regulatory function found in eukaryotes. They can degrade or inhibit the expression and translation of target genes through complementary pairing with the 3' untranslated region (3'UTR) of mRNAs, and are involved in the regulation of cell proliferation, differentiation and individual development (5). Increasing evidence indicates that miRNAs are also important in the development of NSCLC (6-8). The serum miRNAs can be used as key biomarkers for the early diagnosis of NSCLC (9). A numbers of reports have shown that miRNAs are usually localized in tumor-associated fragile sites, where they can affect the occurrence and development of tumors by regulating the corresponding signal pathway as oncogenes or tumor suppressor genes (10). The miRNA (miR)-200 family can be divided into the miR-200a subfamily, including miR-200a and miR-141, and the miR-200 subfamily, including miR200b/c and miR429 (11,12). Studies have shown that the miR-200b/200c/429 subfamily can inhibit the migration of hepatocellular carcinoma cells through affecting the cell cytoskeleton and cell adhesion mediated by Rho/Rho kinase (13). Studies on miR-200 family in lung cancer have mainly focused on their inhibitory effect. A previous study showed that miR-200 suppressed lung adenocarcinoma cell invasion, metastasis and lung tumorigenesis by targeting Fms related tyrosine kinase 1 (14). In addition, miR-200 suppressed cancer cell growth, migration and invasion, and inhibited the tumorigenesis and metastasis of lung cancer cells though downregulating bone morphogenetic protein (BMP4). These findings indicated that BMP4 and miR-200s may be suitable therapeutic targets for the treatment of lung cancer (15). miR-200 is also important in lung cancer resistance. The latest findings have shown that miR-200c may be involved in regulating paclitaxel resistance through Cathepsin L (CTSL)-mediated epithelial-mesenchymal transition (EMT) in A549 cells, and that CTSL and micRNA-200c were reciprocally linked in a feedback loop (16). In addition, the expression of miR-200 was reported to be significantly lower in nintedanib-resistant cell lines than in nintedanib-sensitive cell lines, and the induction of miR-200 enhanced sensitivity to nintedanib in the nintedanib-resistant A549 cells (17). However, the association between RhoE and the miR-200 family in NSCLC remains to be elucidated.

RhoGTPase is a class of 20-30 kDa GTP binding proteins, belonging to the main members of the Ras superfamily (18). Several important findings have been revealed regarding the function of the Rho family members in tumors. RhoGTPase can induce the activation of transcription factors, including c-Jun and nuclear factor-κB through a signal transduction series, causing the reorganization of actin and cell cycle progression or inhibition. Rho family GTPase 3 (RhoE) is a member of the RhoGTPase family, belonging to the RND subfamily, also known as RND3. Unlike other family members, it lacks GTPase activity, so it can only be combined, but cannot be hydrolyzed to GTP. A previous investigation of the mechanism of RhoE showed that it can inhibit RhoA/Rho-associated protein kinase signaling through binding to p190 GTPase-activating protein, which can reduce the levels of RhoA-GTP (19). The majority of studies have shown that RhoE may be a candidate tumor suppressor gene. Previous studies, including ours, have revealed that the tumor suppressor gene p53 can regulate the

activity of RhoE by binding to its promoter (20,21). However, several studies have found that RhoE is relatively overexpressed in NSCLC compared with other tumors. In addition, the expression of RhoE may serve as an unfavorable prognostic factor in patients with NSCLC (22,23). However, the molecular mechanism underling this dysregulation remains to be fully elucidated.

In the present study, it was found that miRNA 200b/c was downregulated, whereas RhoE was upregulated in NSCLC tissues. miRNA 200b/c was found to bind to the RhoE 3'UTR and inhibit its expression. miR-200b/c regulated the migration and invasion of NSCLC cells through directly regulating the expression of RhoE.

Materials and methods

Cell culture. The A549 and NCI-H1299 human NSCLC cells were obtained from the Cell Biology Institute of Chinese Academy of Sciences (Shanghai, China). The A549 cell line was maintained in DMEM (Invitrogen; Thermo Fisher Scientific, Inc., Waltham, MA, USA) with 10% fetal bovine serum (FBS; Gibco; Thermo Fisher Scientific, Inc.), and the NCI-H1299 cell line was maintained in RPMI-1640 medium (Invitrogen; Thermo Fisher Scientific, Inc.) with 10% FBS, 100 U/ml penicillin G sodium, and 100 μg/ml streptomycin sulfate (Sigma; EMD Millipore, Billerica, MA, USA). The cells were cultured in a 37°C incubator containing 5% CO_2.

Plasmids and transfection. The pCDNA3.1-RhoE (pCDNA3.1 was obtained (Invitrogen; Thermo Fisher Scientific, Inc.) and PRL-TK (Promega Corporation, Madison, WI, USA) plasmids were stored in our laboratory. The 3'UTR of the human RhoE gene (NM_001254738) was amplified from human genomic DNA by polymerase chain reaction (PCR), and cloned into the *Xba*I site of the pGL3-control vector (Promega Corporation), following which its sequence was verified and it was termed pGL3-RhoE-wt. The PCR primers used for amplifying the RhoE 3'UTR were as follows: 5'-GCTCTAGACTCCCCTT A AATCGTG-3' (forward) and 5'-GCTCTAGAACTGCGGA AACATTGAC-3' (reverse). In addition, site-directed mutagenesis of the miR-200b/c target site in the RhoE 3'UTR was performed using a site-directed mutagenesis kit (Takara Bio, Inc., Otsu, Japan), with pGL3-RhoE-wt as a template, and termed pGL3-RhoE-mut, respectively. The pGL3-RhoE-mut primers were as follows: 5'-GAACGTACAAAATAGCTGG-3' (forward) and 5'-CCCAAAGCATAATGCTGCAT-3' (reverse). Small interfering (si)RNA sequences against RhoE (Si-RhoE: 5'-AACAGATTGGAGCAGCTACdTdT-3'), control oligo, and hsa-miR-200b/c were constructed and chemically synthesized by Shanghai Genepharma Co., Ltd. (Shanghai, China). All plasmids and RNA oligonucleotides were transfected into cells using Lipofectamine 2000 (Invitrogen; Thermo Fisher Scientific, Inc.) according to the manufacturer's protocol.

Dual-luciferase report assay. The cells ($1x10^5$ per well) were seeded in 24-well plates 1 day prior to transfection. For the dual-luciferase assay, the cells were transfected with 0.3 μg of pGL3-RhoE-wt-luc or pGL3-RhoE-mut-luc plasmids, 60 nM control miRNA or miR-200b/c, together with 0.1 μg PRL-TK in 24-well plates using Lipofectamine 2000

(Invitrogen; Thermo Fisher Scientific, Inc.). The cells were harvested 48 h following transfection and lysed with lysis buffer. Firefly and *Renilla* were detected by the dual-luciferase reporter assay system kit (Promega Corporation). The luciferase activity was determined with the Synergy2 instrument (BioTek Instruments, Inc., Winooski, VT, USA) equipped with Gen5 1.04.5 software. The gene expression was normalized by *Renilla* luciferase (pRL-TK) and reported as relative luciferase activity. Three independent experiments were performed in triplicate. The results are presented as the mean ± standard deviation of data from three dependent experiments.

Reverse transcription (RT)-PCR and quantitative PCR (RT-qPCR) assays. Total RNA from the cells was extracted using TRIzol (Invitrogen; Thermo Fisher Scientific, Inc.) following the manufacturer's protocol. The contaminated genomic DNA was eliminated and total RNA was reverse transcribed into cDNA with the Prime Script RT Reagent kit with gDNA Eraser (Takara Bio, Inc.). Subsequently, PCR was performed on a PCR system (IQ5), using Bio-Rad iQ SYBR-Green Supermix (both from Bio-Rad Laboratories, Inc., Hercules, CA, USA) as follows: A total of 2 μl cDNA template was brought to a volume of 25 μl containing 12.5 μl 2X SYBR premix Ex TaqII, 1 μl of both sense and antisense primer and 8.5 μl H$_2$O. Amplification was performed as the following conditions: One cycle of 95˚C for 30 sec was performed to synthesize the cDNA. Forty cycles of 95˚C for 5 sec, 60˚C for 30 sec for PCR reaction, and a final melt curve analysis of 65˚C for 15 sec.

The primers used for specific RhoE PCR were as follows: Forward, 5'-AAGATAGTTGTGGTGGGAGA-3' and reverse, 5'-CATAGTAAGGAGAACCCGAA-3'. The primers used for specific GAPDH PCR were as follows: Forward, 5'-CAAGGC CAACCGCGAGAA-3' and reverse, 5'-CCCTCGTAGATGG GCACAGT-3'. The primers of miR-200b: Forward, 5'-TAATAC TGCCTGGTAATGATGA-3' and reverse, 5'-ATCATTACCA GGCAGTATAAAT-3'; miR-200c forward, 5'-TAATACTGC CGGGTAATGATGGA-3' and reverse, 5'-CCAGTGCAGG GTCCGAGGT-3'; and U6 forward, 5'-CTCGCTTCGGCAGC ACA-3' and reverse, 5'-AACGCTTCACGAATTTGCGT-3' were synthesized by Genwiz, Inc. (Suzhou, China). The relative expression levels were determined using the 2$^{-\Delta\Delta Cq}$ method (24).

Cell growth assay. The A549 and NCI-H1299 cells were seeded in 96-well plates 24 h prior to transfection. All cells were transfected respectively with control oligos, mimics and siRhoE. Relative cell growth was measured using the Cell Counting Kit-8 (CCK-8; Dojindo Molecular Technologies, Inc., Kumamoto, Japan). Three independent experiments were performed in triplicate.

Cell Transwell migration and invasion assays. At 24 h post-transfection, the A549 cells and H1299 cells were digested by trypsin, and then collected and suspended with serum-free culture medium. The cells (3x10^4) were suspended in 0.2 ml of serum-free culture medium and plated in the top chamber of a membrane insert (Transwell; 24-well insert; pore size 8-μm; BD Falcon; BD Biosciences, Franklin Lakes, NJ, USA). Ten percent FBS medium was placed in the bottom of the 24-well plate as an attractant. The cells were incubated for

48 h, and cells that did not invade through the membrane were removed using a cotton swab. Those cells that had invaded the membrane were stained with crystal violet on the opposite surface of the Transwell for the visualization (Nikon ECLIPSE 2000-U; Nikon, Melville, NY, USA) of invasion. The assays were performed three times.

The cell migration assay was almost the same as the invasion assay, but used the extracellular matrix (1:4, BD Biosciences) Matrigel-coated Transwell. The assays were repeated three times.

Cell cycle analysis. At 48 h post-transfection, the cells were digested by trypsin, and washed three times with ice-cold PBS, and then suspended and fixed with 70% ice-cold ethanol overnight at 4˚C. The fixed cells were rehydrated in PBS and stained with PI/RNase at 37˚C for 15 min following the manufacturer's protocol of the Cell Cycle Detection kit (Nanjing KeyGen Biotech Co., Ltd., Nanjing, China). Cell cycle analyses were performing by fluorescence-activated cell sorting analysis with a flow cytometer (BD FACSCalibur; BD Biosciences).

Western blot analysis. The total protein samples were extracted with RIPA buffer containing a cocktail of protease inhibitors from the transfected cells. The protein samples were quantified by a BCA protein assay kit (Pierce, Rockford, IL, USA). Equal amounts of proteins (30-40 μg) were fractionated by 12% SDS-PAGE (Bio-Rad Laboratories, Inc.), and transferred onto NC membranes (EMD Millipore). The membranes were blocked by 5% skim milk for 1 h and probed with antibodies against human RND3 (1:2,000, 05-723; EMD Millipore), human N-cadherin (1:4,000, 2447-1; Epitomics), E-cadherin (1:1,000, ab1416; Abcam), vimentin (1:4,000, 2862-1), phosphorylated Akt (473; 1:4,000, 2118-1) (both from Epitomics), Akt (1:1,000, ab8805), phosphorylated extracellular signal-regulated kinase (Erk)1/2 (1:2,000, ab214362), Erk1/2 (1:2,000, ab17942) (all from Abcam) and GAPDH (1:1,000, G8795; Sigma; AB2302; EMD Millipore). Fluorescence-conjugated anti-mouseor rabbit IgG (P/N925-32210; P/N925-68071; 1:10,000, LI-COR Biotechnology, Lincoln, NE, USA) were used as the secondary antibodies, and the band intensities were quantified using the LI-COR Odyssey infrared imaging system (LI-COR Biotechnology). The analyses were repeated three times.

Immunohistochemistry (IHC). Human lung adenocarcinoma tissues were obtained from the Disease Tissue Specimen Bank of West China Hospital, Sichuan University, from 40 cases of lung carcinoma, including normal control and tumor tissues. All patients signed an informed consent before tissue collection by the Disease Tissue Specimen Bank of West China Hospital, and the study was approved by the Ethics Committee of Sichuan University. The clinicopathological grades of all tissues were evaluated using World Health Organization standards. The IHC experiments were performed using the standard avidin-biotin complex immunoperoxidase method. The slides were incubated with anti-RhoE monoclonal antibody (1:200; Abcam; ab79999), human N-cadherin (1:200; Epitomics; 2447-1), E-cadherin (1:100; Abcam; ab1416), vimentin (1:200; Epitomics; 2862-1). PBS was used as a negative control instead of RhoE antibody. The ratio of positive cells per specimen was
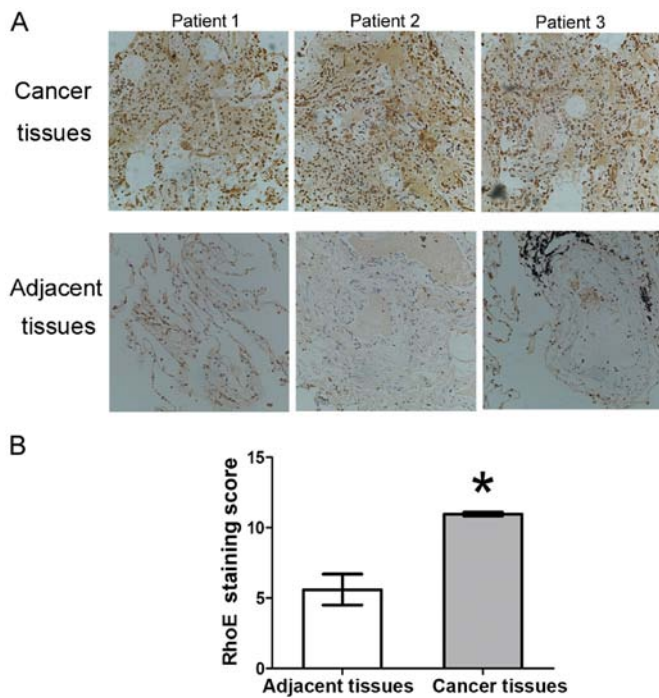
A

B

Figure 1. Expression of RhoE is upregulated in human lung adenocarcinoma tissues. (A) Epithelial cells representing lung adenocarcinoma tissues showed weak immune signals (bottom panels, magnification, x20; 10X objective). The corresponding lung adenocarcinoma tissues showed strong immunosignals (upper pnales, magnification, x20; 10X objective). (B) Average staining scores of RhoE in lung adenocarcinoma tissues and adjacent tissues (*P<0.05). RhoE, Rho family GTPase 3.

Table I. Clinicopathological features of patients with non-small cell lung cancer.

Clinical characteristic	Total number
Age (years)	
>60	20
≤60	20
Sex	
Male	26
Female	14
Invasion depth	
T1	10
T2	29
T3	1
T4	0
Lymph node metastasis	
0	16
1	7
2	17
3	0
Metastasis	
Yes	2
No	38
TNM stage	
IA	4
IB	10
IIA	7
IIB	0
IIIA	17
IV	2

TNM, tumor-node-metastasis.

evaluated quantitatively and scored using the thirteen score method: 0 for staining ≤1%, 1 for staining of 2-25%, 2 for staining of 26-50%, 3 for staining of 51-75%, and 4 for staining >75% of the cells examined. Staining intensity was graded as follows: 0, no signal; 1, weak; 2, moderate; and 3, strong staining. The two scoring systems were utilized to evaluate the levels of protein expression and a total score of 0-12 was finally calculated and graded (20).

Bioinformatics analyses. Bioinformatic analysis was performed for searching putative miRNAs predicted to target RhoE coding or non-coding sequences using PicTar (https://pictar.mdc-berlin.de).

Statistical analysis. A paired-samples t-test was used to compare the staining scores of the IHC samples. Characterizing the phenotypes of cells were analyzed using one-way analysis of variance(ANOVA) and Dunnett's post test. Data analysis was performed using SPSS for Windows version 14.0 (SPSS, Inc., Chicago, IL, USA). The test level of α was 0.05. P<0.05 was considered to indicate a statistically significant difference.

Results

IHC analysis of the expression of RhoE in human lung adenocarcinoma tissues. In our previous study, it was shown that the expression of RhoE was decreased to different degrees in gastric cancer, breast cancer and colorectal cancer tissues, and its expression was likely regulated by epigenetic modification (20).

In order to examine the protein expression of RhoE in human lung adenocarcinoma tissues, 40 lung adenocarcinoma tissues were matched with adjacent normal tissues, and the tissue information of patients, including age, gender, tumor size, distance-metastasis and more advanced tumor-node-metastasis stage, are shown in Table I. IHC was used to detect the expression of RhoE in the tissues, and the results showed that RhoE was expressed at a higher level in tumor tissues than that in the adjacent normal tissues (Fig. 1A). RhoE was expressed in the tissues of all 40 cases of lung adenocarcinoma and the average staining score was 10.91±0.18 (positive ratio of 100%), whereas the average staining scores of the corresponding adjacent tissues was 5.86±1.27 (positive ratio of 60%). There were significant differences in the staining score and positive rate between the tumor tissues and adjacent tissues (P<0.005) (Fig. 1B). Therefore, RhoE was overexpressed in the human lung adenocarcinoma tissues.

RhoE 3'UTR contains a putative target site for miR-200b/200c, which is conserved among species and is a direct target of miR-200b/200c. Studies have shown that miRNAs can regulate
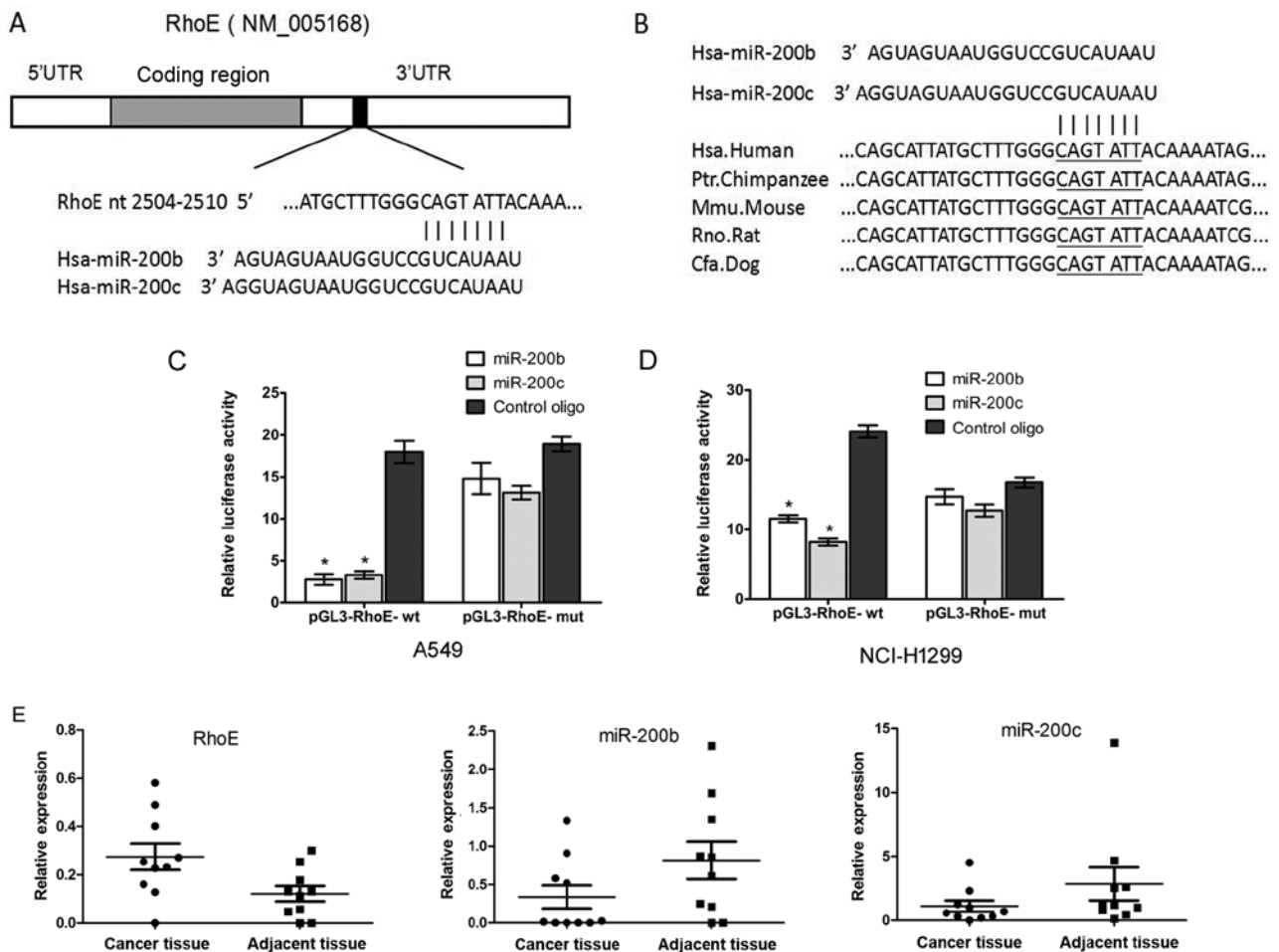
Figure 2. 3'UTR of RhoE contains a putative target site for miR-200b and miR-200c that is conserved across species. (A) Putative binding sites targeted with RhoE 3'UTR and miR-200b/200c are shown in the schematic representation. (B) Nucleotide sequences of the miR-200b/200c seed sequences and its target site across different species. Sequence analysis suggested that miR-200b/200c target sequence at nt 2504-2510 (CAGUAUU) of the RhoE 3'UTR was conserved among different species. The target site for miR-200b/200c within the RhoE 3'UTR is underlined. pGL3-RhoE-wt or pGL3-RhoE-mut luciferase constructs containing a wild-type or a mutated RhoE 3'UTR were cotransfected into (C) A549 and (D) NCI-H1299 cells with control mimics or miR-200b/200c, respectively. Luciferase activity was determined 48 h following transfection. The ratio of normalized luciferase activity is shown. Data were obtained from three independent experiments performed in triplicate. *$P<0.05$ vs. control miR-transfected cells. (E) Reverse transcription-quantitative polymerase chain reaction analysis of the expression of miR-200b/c and RhoE in NSCLC tissues and the paired adjacent tissues. The expression of miR-200b/c was normalized to that of U6 using the $2^{-\Delta\Delta Cq}$ method. Bars represent the standard deviation. Data are representative of three independent experiments performed in triplicate. RhoE, Rho family GTPase 3; 3'UTR, 3' untranslated region; miR, microRNA; NSCLC, non-small cell lung camcer.

cell apoptosis and drug sensitivity through directly targeting RhoE. To examine and confirm the regulation of RhoE by miRNAs, the bioinformatics tool PicTar was used to search for putative miRNAs that are predicted to target the RhoE gene (25). Finally, hsa-miR-200b/200c was focused on as putative miRNA binding to the 3'UTR of human RhoE, and sequence analysis revealed that the targeting sequence for miR-200b/200c is located at nt 2504-2510 of the RhoE 3'UTR (Fig. 2A), and this region is conserved across different species, as shown in Fig. 2B.

To confirm whether RhoE was a direct target of miR-200b/200c, a dual-luciferase reporter assay was performed to verify the sequence regions of the RhoE-3'UTR that were responsible for the direct regulation of miR-200b/c. The 3'UTR of the human RhoE gene was cloned into the pGL3-luciferase reporter vector (pGL3-control) to determine whether it is a direct functional target of miR-200b/c. This recombinant plasmid was termed pGL3-RhoE-wt. In parallel, another luciferase reporter construct was generated, in which the putative miR-200b/c targeting region CAGTATT located within

nt 2504-2510 was specifically mutated as pGL3-RhoE-mut. Transient transfection with pGL3-RhoE-wt and miR-200b/c led to a significant decrease of luciferase activity compared with the control in A549c cells (Fig. 2C) and NCI-H1299 cells (Fig. 2D). Mutation of the miR-200b/c putative binding site in the RhoE-3'UTR eliminated the inhibitory effect on luciferase expression by miR-200b/c. These results indicated that miR-200b/c directly targeted the expression of RhoE through binding to the predicted sequence sites.

To compare the expression of miR-200b/200c and RhoE in lung adenocarcinoma tissues and adjacent tissues, the mRNA expression levels of miR-200b, miR-200c and RhoE in lung adenocarcinoma tissues and paired adjacent samples were detected using RT-qPCR analysis. The results showed that the mRNA expression of RhoE in the tumor tissues was higher than that of the adjacent tissues, whereas the mRNA expression levels of miR-200b and miR-200c in the lung adenocarcinoma cancer tissues were significantly lower than those in the adjacent tissues (Fig. 2E).
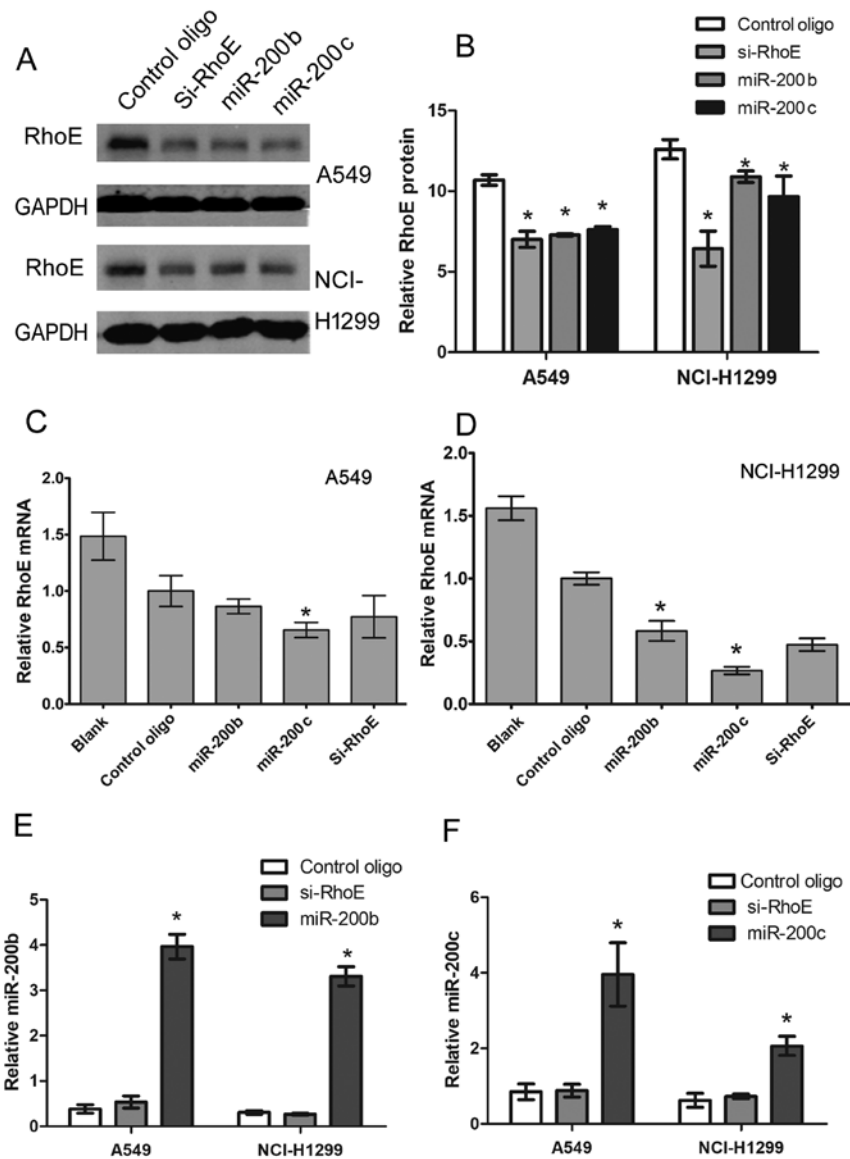
Figure 3. Protein and mRNA expression of RhoE is regulated by miR-200b/200c. (A) Protein expression of RhoE in A549 and NCI-H1299 cells 48 h following transfection with miR-200b/200c, Si-RhoE or control oligo, determined with western blot analysis. (B) Band intensities were quantified with the Odyssey infrared imaging system. Data are presented as the mean ± standard deviation from three independent experiments. mRNA expression of RhoE in (C) A549 and (D) NCI-H1299 cells 48 h following transfection with miR-200b/c, Si-RhoE or control oligo, determined by RT-qPCR analysis. mRNA expression of RhoE was normalized to mRNA expression of GAPDH, and data are shown as a ratio of miR200b/200c and Si-RhoE-transfected cells to control oligo-transfected cells using the $2^{-\Delta\Delta Cq}$ method. Data are presented as the mean ± standard deviation from three independent experiments. *P<0.05 vs. control oligo-transfected cells. RT-qPCR analysis of (E) miR-200b and (F) miR-200c in A549 and NCI-H1299 cells transfected with miR-200b/c, Si-RhoE or control oligo. Expression of miR-200b/c was normalized to the expression of U6 using the $2^{-\Delta\Delta Cq}$ method, Data are representative of three independent experiments performed in triplicate. RhoE, Rho family GTPase 3; RT-qPCR, reverse transcription-quantitative polymerase chain reaction; miR, microRNA; Si-small interfering RNA.

miR-200b/c can affect the expression of RhoE in NSCLC cell lines. To examine whether miR-200b/c can affect the expression of RhoE in NSCLC cell lines, the A549 and NCI-H1299 cell lines, which show a relatively high expression of RhoE, were transfected with miR-200b/c, a control oligo or Si-RhoE mixture targeting RhoE. The protein and mRNA expression levels of RhoE were analyzed by western blot and RT-qPCR analyses, respectively. As shown in Fig. 3, compared with the negative control, transient expression of miR-200b/c led to a significant decrease of RhoE at the protein (Fig. 3A and B) and mRNA (Fig. 3C and D) levels, similar to that caused by transfection with Si-RhoE in the two cell lines. Furthermore, RT-qPCR analysis was performed to detect the expression of miR-200b/c in the transfected cell lines, the results of which showed that miR-200b/c was successfully overexpressed (Fig. 3E and F). Based on these data, it was concluded that RhoE was negatively regulated by miR-200b/c, and the 3'UTR of RhoE was a functional target site for miR-200b/c in the NSCLC cells.

miR-200b/c inhibits proliferation, migration, invasion and induces G0/G1 arrest in NSCLC cells. RhoE is key in the regulation of cell cycle progression at the G1/S transition (26). The present study investigated the effects of miR-200b/c and RhoE siRNA on cell proliferation. The results showed that the ectopic expression of miR-200b/c markedly inhibited the proliferation of A549 (Fig. 4A) and NCI-H1299 (Fig. 4B) cells in the CCK-8 assays. This effect was comparable to that of
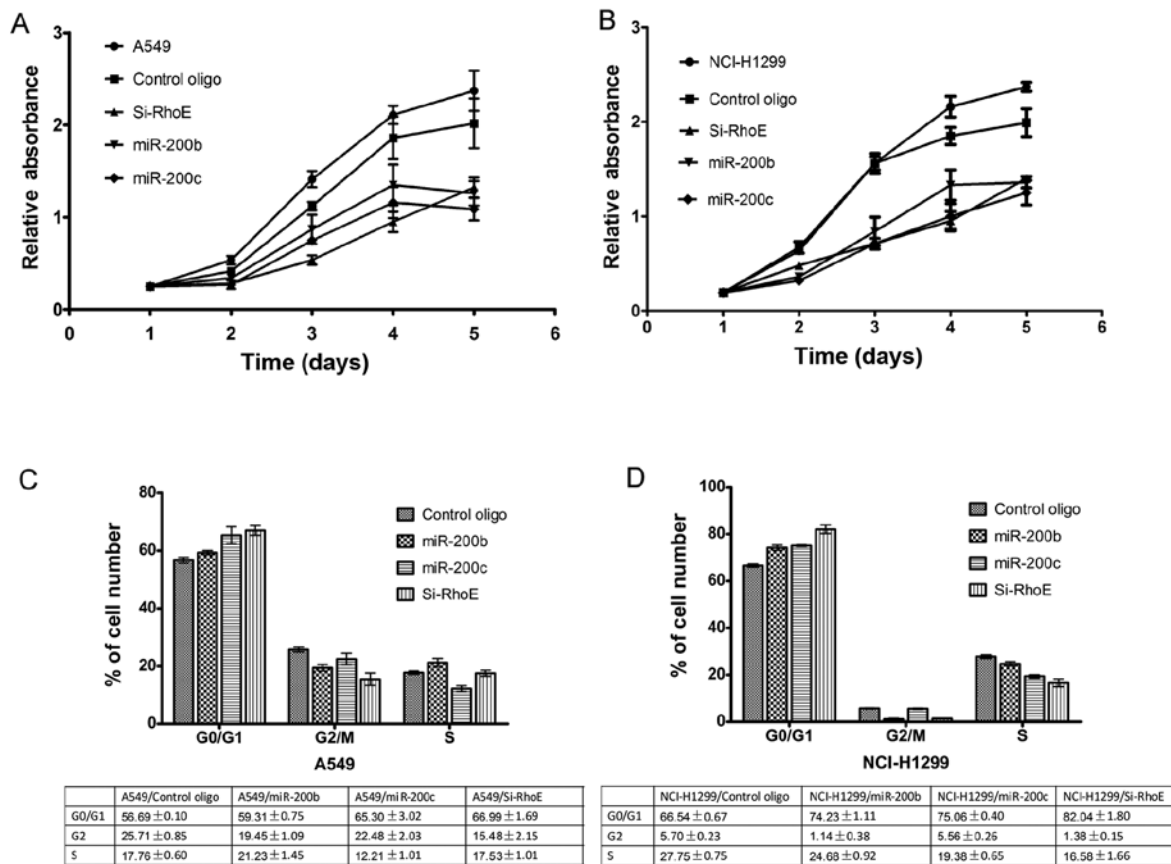
Figure 4. miR-200b/200c inhibits the proliferation potential of NSCLC cells. Proliferation potential of (A) A549 and (B) NCI-H1299 cells 24 h following transfection with miR-200b/200c, Si-RhoE or Control oligo, determined using the cell counting kit-8 kit assay. The data are presented as the mean ± standard deviation from three independent experiments performed in triplicate. miR-200b/200c induced cell cycle G1 arrest in NSCLC cells. Cell cycle progression of (C) A549 and (D) NCI-H1299 cells 48 h following transfection with miR-200b/200c, Si-RhoE or Control oligo, determined by fluorescence-associated cell sorting analysis. Data are representative of three independent experiments. NSCLC, non-small cell lung cancer; RhoE, Rho family GTPase 3; miR, microRNA; Si-small interfering RNA.

RhoE siRNA. To further examine the underlying mechanisms, the cell cycle distributions of the miR-200b/c or RhoE siRNA transfectants were analyzed by FACS analysis. The cell cycle progression of A549 (Fig. 4C) and NCI-H1299 (Fig. 4D) cells transfected with miR-200b/c or the siRNA mixtures was arrested at the G1 phase, with a concomitant decrease of cells in the S and G2/M phases, compared with cells transfected with control oligos. These results indicated that miR-200b/c inhibited the G1/S phase transition by downregulating RhoE. In addition to the regulation of cell proliferation, RhoE is involved in the migration and invasion of tumor cells, contributing to the incidence and mortality rates of patients with cancer. In order to determine whether miR-200b/c affects cell invasion, Matrigel invasion and migration assays were performed. The results showed that miR-200b/c significantly decreased the invasion and migration activity of the two cells, similar to the effect of siRNA mixtures of transfected cells (Fig. 5). These data suggested that miR-200b/c negatively regulated the invasive ability of NSCLC cells, at least in part through RhoE-mediated targeting activity.

Expression of EMT-related transcriptors is correlated with miR-200b/c and RhoE in NSCLC cells. To further examine the regulatory mechanism of miR-200b/c-RhoE in NSCLC cells, the A549 and NCI-H1299 cells were transfected with miR-200b/c, RhoE, or control oligo, and the expression levels of AKT, ERK1/2 and EMT-related markers, including N-cadherin, E-cadherin and vimentin, were examined by western blot analysis. As shown in Fig. 6A-C, the expression of E-cadherin was increased, and the expression of N-cadherin and vimentin were decreased significantly in the cells over-expressing miR-200b/c, and the same effect was detected in those cells with RhoE knockdown. In addition, the expression of EMT-related proteins N-cadherin, E-cadherin and vimentin were examined in human lung adenocarcinoma tissues. The results showed that the expression of E-cadherin was relatively low and that of N-cadherin/vimentin was relatively high (Fig. 6D and E) in the cancer tissues, compared with that in the adjacent tissues. However, no significant change, or even the opposite expression pattern, was observed in the expression of AKT, p-AKT, ERK1/2 or p-ERK1/2 in the miR-200b/c group or siRhoE group compared with the controls. Together, the above data indicated that miR-200b/c may also act through RhoE to regulate the EMT-related markers, although the exact mechanisms require further investigation.

Discussion

miRNAs, a group of small, non-coding RNA molecules, act as post-transcriptional gene expression regulators in several
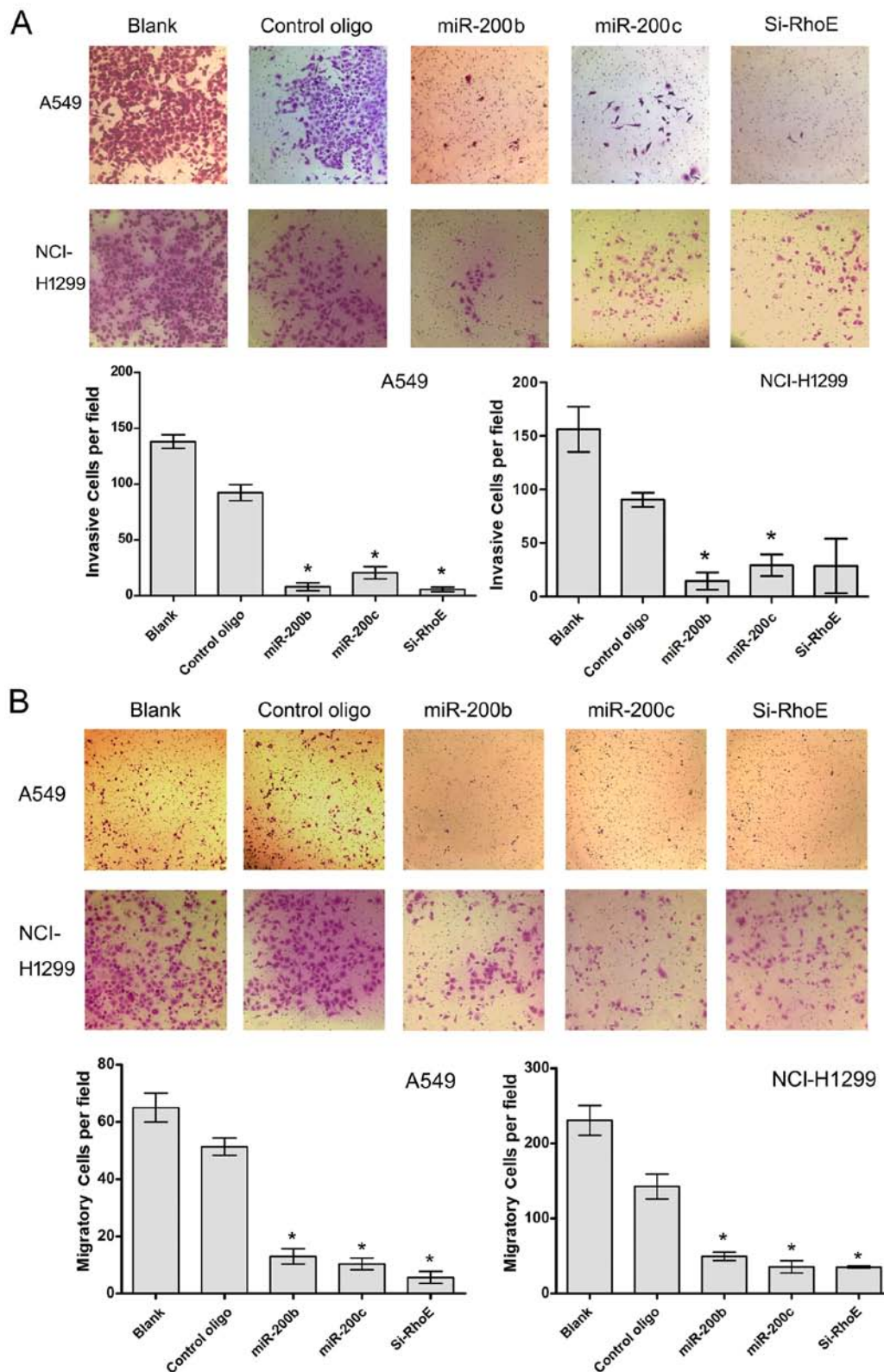
Figure 5. miR-200b/200c inhibits non-small cell lung cancer cell invasion and migration. (A) Invasive activities of A549 and NCI-H1299 48 h following transfected with miR-200b/200c, Si-RhoE or control oligo, were assayed with a Matrigel-coated Transwell. (B) Migration activities of A549 and NCI-H1299 48 h following transfection with miR-200b/200c, Si-RhoE or control oligo, were assayed in a Transwell without Matrigel, and cells that successfully invaded and migrated were stained and quantified 48 h following plating. Data are representative of three independent experiments (magnification, x20; 10X objective). *P<0.05 vs. control oligo-transfected cells. RhoE, Rho family GTPase 3; miR, microRNA; Si-small interfering RNA.

important physiological processes, including development, cell differentiation and carcinogenesis. In previous years, increasing evidence has supported the fact that miRNAs can function as oncogenes or tumor suppressor genes in the regulation of multiple tumorigenesis (27). The miR-200 family, reported as 'new star' miRNAs, consist of five members and are key in a
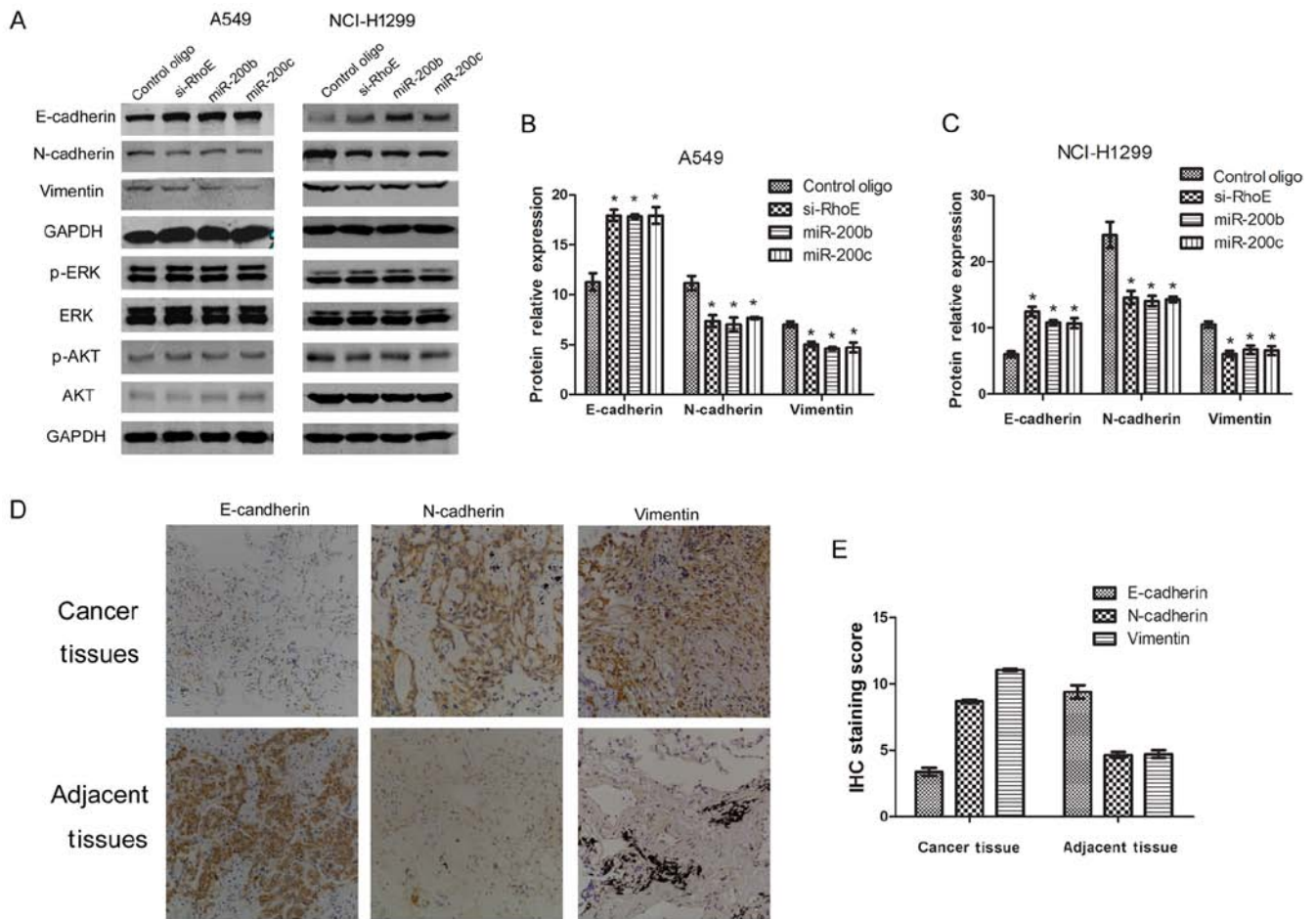
Figure 6. EMT-related protein expression. (A) Overexpression of miR-200b/200c induces EMT signaling changes similar to that induced by knockdown of RhoE. At 48 h post-transfection with miR-200b/200c, Si-RhoE or control oligo in A549 and NCI-H1299 cell line 48 h, E-cadherin, N-cadherin, vimentin, ERK1/2, p-ERK1/2, AKT and p-AKT were examined by western blot analysis. Band intensities in (B) A549 and (C) NCI-H1299 cells were quantified with the Odyssey infrared imaging system. Data are presented as the mean ± standard deviation from three independent experiments. (D) Expression of EMT signaling pathway-related molecules in human lung adenocarcinoma tissues (magnification, x20; 10X objective). (E) Average staining score of EMT-related proteins in lung adenocarcinoma tissues and adjacent tissues ($^{*}P<0.05$ vs. control). RhoE, Rho family GTPase 3; miR, microRNA; Si-small interfering RNA; EMT, epithelial-mesencyhmal transition; ERK, extracellular signal-regulated kinase; p-, phosphorylated.

series of important physiological processes in cancer, including the modulation of cell division and apoptosis, repression of cancer stem cells and reversal of chemoresistance (28). miR-200s are involved in the inhibition of EMT by targeting of transcriptional repressors of E-cadherin and Zinc finger E-box binding homeobox 1 (ZEB1) in a negative feedback loop (29) and serving as a key indicator of E-cadherin-positive and vimentin-negative cancer cell lines (12,30). miR-200s were found to decrease the expansion of human metastatic prostate cancer cells through inhibiting the notch signaling pathway (31). In addition, miR-200s can serve as a prognostic marker for patients with cancer (32,33). It was found that the loss of miR-200c induced significant inhibition of the invasion and migration in NSCLC cells. In previous data, a gene set enrichment analysis performed using the Cancer Cell Line Encyclopedia database showed that miR-200c was suppressed, the expression level of E-cadherin was downregulated, and the expression levels of vimentin and ZEB1 were overexpressed among 34 NSCLC cell lines (34). It was reported that miR-200c acted as an antitumor gene in cells with acquired EGFR-TKI resistance (35), and that no miR-200c was expressed in cancer stem cells (36). To date, these results suggest that the deregulation of miR-200 is key in multiple levels

as a tumor suppressor gene. In contrast to the results of the above studies, the results of a clinical tissue microarray showed that high expression levels of tumor miR-200c were correlated with poor prognosis in patients with lung cancer (32), and a high level of miR-200c has been found in ovarian (37), cervical (36), bile duct (38) and nasopharyngeal (39) cancer. These results indicate that the role of miR-200c in cancer remains controversial; in terms of the mechanism, it may be that miR-200s has a different role in the progression of different cancer types. However, the regulatory mechanism of miR-200 in NSCLC remains to be fully elucidated.

In the present study, it was found that miR-200b/c regulated the proliferation, invasion and migration in NSCLC cell lines by targeting the Rho family protein RhoE. RhoE is a unique and atypical member of the RND subfamily and RAS superfamily, which regulates a series of cell activities and disease progression (40). Compared with other typical Rho family members, RND proteins are atypical in terms of the protein structures and functional binding, but do not hydrolyze GTP, thus retaining constitutive activity, which is not regulated by guanine nucleotide exchange factor (GEF) or GTP-activating protein (GAP) (41). In addition, the carboxy-terminal sequence

of RhoE is farnesylated, whereas the majority of Rho family proteins are modified by geranylation. Therefore, the activity of RhoE is not regulated by GEFs, GAPs or GDP-dissociation inhibitors, the typical regulators, but by regulating the balance between transcription, translation and degradation, and by post-translational modifications including phosphorylation (42). These results indicated that the regulation model of RhoE may be different from other Rho family members (41).

In a previous study, RND3 was overexpressed at mRNA and protein levels in NSCLC, and was positively correlated with the unfavorable prognosis of patients. This suggested that the expression of RND3 may be investigated as a marker of prognosis in patients with NSCLC (19). In order to translate and develop for the clinical application of NSCLC biomarkers, selection in a large cohort of patients was established by the long-term survival rates of patients and the expression of several signatures, including RhoE, in the complete resection of lung adenocarcinoma (43). The findings provided evidence for the overexpression of RhoE, which was associated with poor prognosis and tumor progression in patients with NSCLC as a marker for clinical prognosis, however, this remained to be validated in a prospective study (44). Previous studies have shown that RhoE was downregulated in three NSCLC cell lines, and inhibited cell proliferation through the Notch1/Notch intracellular domain/Hes1 signaling pathway (45). However, only one cell line was obtained from lung adenocarcinoma, which was a limitation of this study. In addition, the mechanism of the upregulation in NSCLC remained unclear. Therefore, evaluation of the impact of the dysregulation of RhoE on NSCLC requires further investigation.

In the present study, RhoE was overexpressed in NSCLC, which was different from other types of tumor. It was found that human RhoE harbored two putative sites recognized by miR-200b and miR-200c, which suggested that miR-200b/c may be directly regulated by RhoE at the post-transcription level. A dual-luciferase-based reporter assay confirmed the binding sites of miR-200b/c on the 3'UTR of RhoE, and miR-200b/c inhibited the protein and mRNA expression levels of RhoE. miR-200b/c significantly inhibited proliferation and invasion activities and induced G0/G1 arrest in the A549 and NCI-H1299 NSCLC cells. Previous studies have shown that miR-200b/c is involved in the regulation of EMT, which can be used in tumor diagnosis and as a treatment target, however, the specific regulatory mechanism remained to be elucidated. In the present study, the suppression of RhoE and overexpression of miR-200b/c had a similar effect on the EMT signaling pathway, indicating that miR-200b/c regulated EMT and that this regulation may be regulated by RhoE.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

QT and HX designed the experiments. QT performed the majority of the experiments and analyzed the data. QT and HX wrote the manuscript. ML carried out the construction of plasmids and performed Transwell assay. LC performed the real-time PCR assay. ML, LC and HX helped to revise the manuscript. All authors have read and approved the final manuscript.

Ethics approval and consent to participate

All patients signed an informed consent when collecting the pathological tissue by Disease Tissue Specimen Bank of West China Hospital, Sichuan University and the study was approved by the Ethics Committee of Sichuan University.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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