Function of miR-200a in proliferation and apoptosis of non-small cell lung cancer cells

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Abstract. Lung cancer is the most prevalent type of cancer worldwide and is the leading cause of cancer-associated cases of mortality in the USA and China. Non-small cell lung cancer (NSCLC) accounts for 80-85% of lung cancer cases. microRNAs (miRs) serve multiple roles in the pathogenesis of lung cancer. The current study investigated the lower level of miR-200a in tumor tissues compared with healthy tissue. Overexpression of miR-200a inhibited NSCLC cell proliferation and promoted apoptosis. miR-200a was identified to target Rhophilin Rho GTPase binding protein 2 (RHPN2) and higher levels of RHPN2 were observed in tumor tissues compared with adjacent normal tissues. The current study proposes that miR-200a exhibits a tumor suppressive role in NSCLC and suggests that miR-200a could target RHPN2.

Introduction

Lung cancer is the most prevalent cancer type worldwide (1,2) and is the leading cause of cancer-associated cases of mortality in the USA and China (3). The American Cancer Society estimates that 159,260 Americans succumbed to lung cancer in 2014, which accounts for approximately 27% of all cancer-associated cases of mortality (4). In China, the estimated number of lung cancer-associated cases of mortality was 61,200 in 2015 (5). Despite vigorous efforts to prevent and treat this deadly disease, the 5-year survival rate of lung cancer (16.6%) is lower than numerous other cancer types, including colon (64.2%), breast (89.2%) and prostate cancer (99.2%) (4).

Non-small cell lung cancer (NSCLC) accounts for 80-85% of all cases of lung cancer (6). A number of patients with NSCLC are diagnosed at an advanced stage and curative surgical resection is only suitable for a limited number of cases (7). Overall, more than half of patients with NSCLC succumb to the disease within 1 year of being diagnosed (7). There is an urgent need to develop new strategies for treatment to lower the lung cancer mortality rate. MicroRNAs (miRNAs or miRs) serve multiple roles in the pathogenesis of lung cancer (8-14). MiR-200a, which belongs to the miR-200 family, is important for the initiation and invasion of tumors (15-20). A previous study has identified that miR-200a targets epidermal growth factor receptor and c-Met to inhibit migration, invasion and gefitinib resistance in NSCLC (19). Additionally, miR-200a may be a prognostic marker in metastatic NSCLC cells (20). However, the role of miR-200a in NSCLC is not fully understood.

The current study examined the expression of miR-200a in lung cancer tissues and assessed the function of miR-200a in NSCLC cells *in vitro*.

Materials and methods

Tissue samples. A total of 12 paired NSCLC and adjacent normal tissue samples were obtained from the Department of Pathology, West China Hospital of Sichuan University (Sichuan, China) between September 2014 and May 2016 (age range, 49-66 years; male:female ratio, 7:5). The use of human tissues was evaluated and approved by the Ethical Committee of Sichuan University. Written informed consent was obtained from all patients. All specimens were handled with no knowledge of the patients' identities, as legally required in China. The NSCLC tissue samples were evaluated and confirmed by a senior pathologist at the Sichuan University Cancer Center (Sichuan, China).

Cell culture. The NSCLC cell lines, H1703 and H2172, and the normal lung cell line, HEL299, were purchased from the Cell Bank of Sichuan University (Sichuan, China). Cells were cultured in Dulbecco's modified Eagle's medium (Thermo Fisher Scientific, Inc.) and supplemented with 10% fetal bovine serum (Thermo Fisher Scientific, Inc.). Cell were maintained at 37˚C and 5% CO_2.

Immunohistochemical (IHC) analysis and point scoring system. A total of 12 paired NSCLC and adjacent normal tissue samples were processed using a standard protocol for IHC analysis. Briefly, the tissues were fixed in 10% neutral buffered formalin at room temperature for two days and

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embedded in paraffin. Four-micrometers thick sections were cut. For antigen retrieval, deparaffinization and hydration were performed. The slides were incubated overnight with anti-Rhophilin Rho GTPase binding protein 2 (RHPN2; catalog no. PA5-62469; 1:1,000; Thermo Fisher Scientific, Inc.) at room temperature. Following three washes with PBS, the slides were incubated with goat anti-rabbit IgG polyclonal horseradish peroxidase-conjugated secondary antibody (catalog no. 32260; Thermo Fisher Scientific Inc.; 1:1,000) at room temperature for 2 h. The slides were developed by staining with diaminobenzidine (A600885; Sangon Biotech Co., Ltd.) and quantified manually (21). The slides were subsequently observed using a light microscope (Eclipse E100; Nikon Corporation; magnification, x200). IHC staining was scored according to the following criteria: - (0-10% of the nucleated cells stained); + (10-40% stained); ++ (40-70% stained) and +++ (70-100% stained).

Detection of miR-200a and RHPN2 by reverse transcription-quantitative polymerase chain reaction (RT-qPCR). Total RNA was extracted from the NSCLC tissue samples and cell lines using TRIzol reagent (Thermo Fisher Scientific, Inc.) according to the manufacturer's protocol. The level of miR-200a was measured using the Taqman miRNA RT-Real Time PCR kit (Thermo Fisher Scientific, Inc.) (22). qPCR was performed using the following primers: RHPN2 forward, 5'-AAGGGCTGTAA TCCCCTTGC-3' and reverse, 5'- CCGCACCTTTGAGTTTG TGG-3'; and GAPDH forward, 5'GAAGGTGAAGGTCGGA GTC-3' and reverse, 5'GAAGATGGTGATGGGATTTC-3'. The primer sequences were as following: miR-200c forward, 5'-CGTAATACTGCCGGGTAATGAT-3' and reverse, 5'-GTGTCGTGGAGTCGGCAA-3' and U6 forward, 5'-GCTTCGGCAGCACATATACTAAAAT-3' and reverse, 5'-CGCTTCACGAATTTGCGTGTCAT-3'. PCR thermocycling conditions for RHPN2 and GAPDH were as follows: 95˚C for 10 min; 42 cycles at 95˚C for 15 sec and 60˚C for 1 min. PCR thermocycling conditions for miR-200a and U6 were as follows: 40 cycles of three-step PCR (95˚C for 15 sec, 55˚C for 30 sec and 72˚C for 30 sec) following initial denaturation at 95˚C for 10 min. Quantification was performed using the $2^{-\Delta\Delta Cq}$ method (23).

Prediction of the putative targets of miR-200a. TargetScan software (http://www.targetscan.org/) was used to predict the putative targets of miR-200a (24-26).

Cell proliferation assay. Cellular proliferation was analyzed by a MTT assay. Briefly, H1703 and H2172 cells were placed into 96-well plates at $5x10^3$ cells/well. MTT reagent was added at a final concentration of 0.1 mg/ml with 100 μl dimethyl sulfoxide at 24, 48 and 72 h. The optical density was measured on a microplate reader at a wavelength of 570 nm (27).

Cell apoptosis analysis. The cell apoptosis rate was analyzed using the Alexa Fluor 488 Annexin V/Dead Cell Apoptosis Kit (Thermo Fisher Scientific, Inc.). Briefly, cells ($5x10^5$/ml) were suspended in Annexin V binding buffer (Thermo Fisher Scientific, Inc.) and stained with Annexin V-FITC and incubated for 15 min at room temperature. The cells were subsequently stained with propidium iodide (PI) at room temperature for 15 min (1:1,000; Thermo Fisher Scientific, Inc.). The samples were analyzed using a flow cytometer with a 488 nm excitation laser (argon-ion or solid state) and emission was detected at 530 nm (green; for FITC) and 575-610 nm (orange; for PI) with BD FACSuite software (version 1.0.4.2650; BD Biosciences).

Overexpression and downregulation of miR-200a in H1703 and H2172 cells. miR-200a expression levels in H1703 and H2172 cells were increased and decreased by transfection with a miR-200a mimic and miR-200a antisense oligonucleotide (ASO) (both from Aksomics; ArrayStar), respectively. Cells transfected with miR-negative control (NC) or miR-NC-ASO were used as controls. Prior to transfection, the cells were cultured overnight ($1x10^6$/well). Cell transfection was performed with Lipofectamine™ 2000 (Invitrogen; Thermo Fisher Scientific, Inc.) according to the manufacturer's protocol. The sequences of the miR-200a mimic, the miR-200a AOS, the miR-NC and the miR-NC-ASO were as follows: miR-200a mimic, UAACACUGUCUGGUAACGAUGU; miR-200a ASO, ACAUCGUUACCAGACAGUGUUA; miR-NC, CAG AUUUUGUGUAGUACAA; and miR-NC-ASO, ACTGCCT GTCTGTGCCTGCTGT.

Western blot analysis. Cells were frozen and lysed in lysis buffer (150 mM NaCl, 50 mM Tris-HCI, 1% Triton X-100 and 0.1% sodium dodecyl sulfate) with a protease inhibitor cocktail (Sigma-Aldrich; Merck KGaA). The protein levels were determined by the bicinchoninic acid assay (28) and 30 μg total protein/lane were separated via SDS-PAGE on a 10% gel (100 V for 2 h). Proteins were subsequently transferred from the gel onto a methanol-activated polyvinylidene difluoride membrane. The membrane was blocked for 1 h at room temperature using 5% milk. To detect RHPN2 protein, the membrane was incubated with anti-RHPN2 antibody (cat. no. ab23148; 1:1,000; Abcam) overnight at 4˚C. Following primary antibody incubation, the membrane was incubated a peroxidase-linked secondary antibody (1:2,000; cat. no. ab97120; Abcam) at room temperature for 2 h. To detect β-actin protein, anti-β-actin antibody (1:1,000; cat. no. ab179467; Abcam) was used at a dilution of 1:1,000, followed by detection with a peroxidase-linked antibody to rabbit antibody IgG (1:2,000 dilution; cat. no. ab6747; Abcam). Proteins were detected with the ECL Western Blotting Detection reagents (GE Healthcare). Images were analyzed using ImageJ version 2 FX software (National Institutes of Health).

Dual-luciferase reporter assays. Cells were seeded at 1x105 per well and serum-starved for 6 h prior to transfection. An RHPN2 3'-untranslated region (3'UTR) mutant was generated using a site-directed mutagenesis kit (Promega Corporation). The mutated control was cloned and inserted into a pGL3-control vector (100 ng; Promega). The wild-type and the mutated 3'UTR of RHPN2 was cloned and inserted into a firefly luciferase reporter plasmid (pGL3 luciferase reporter vector; Promega Corporation). A miR-200a mimic and the reporter plasmid or the mutant 3'UTR plasmid (500 ng) were then co-transfected into H1703 cells with Lipofectamine® 2000 (Invitrogen; Thermo Fisher Scientific, Inc.). Cells were harvested and luciferase activity was measured after 24 h using the Dual-Luciferase Reporter assay system (Sangon Biotech Co., Ltd.).

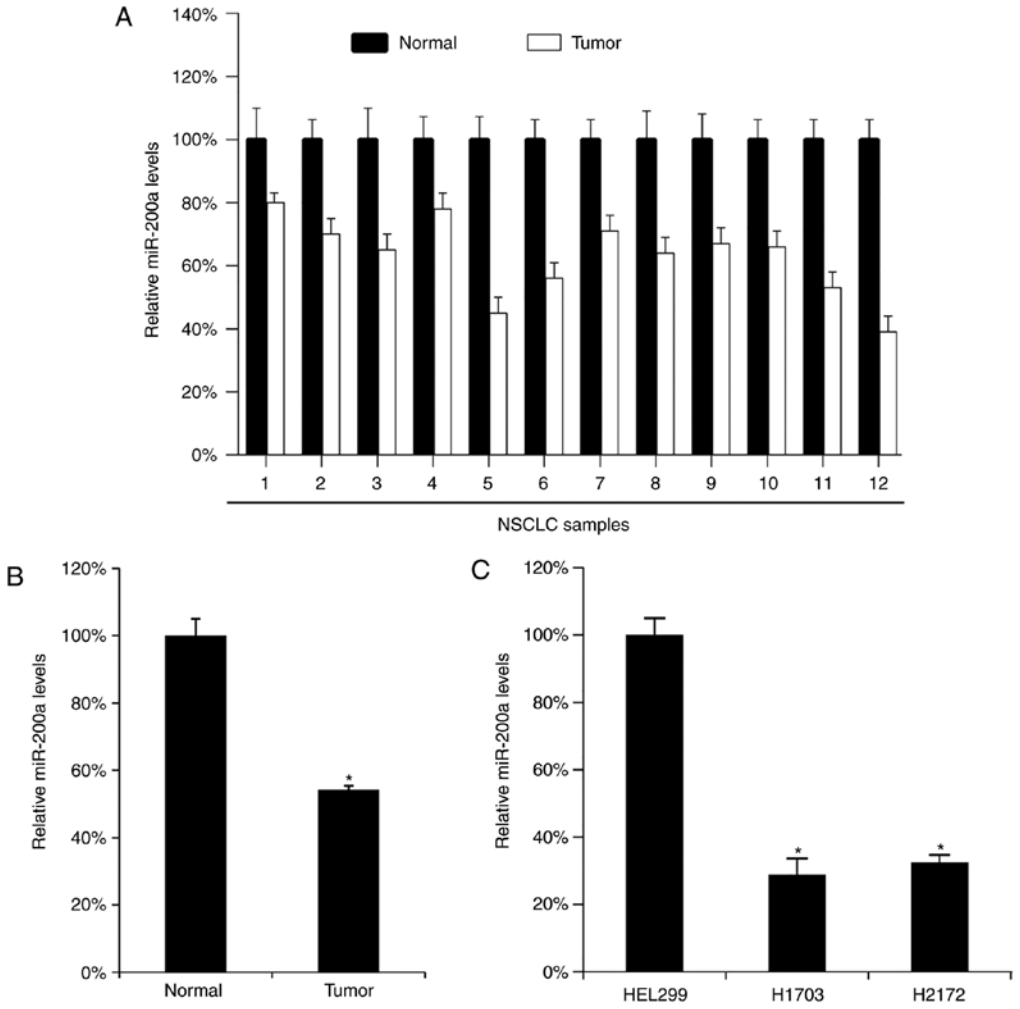

Figure 1. NSCLC tissue samples exhibit low levels of miR-200a. (A) A total of 12 paired NSCLC and adjacent normal tissue samples were collected from West China Hospital of Sichuan University. The miR-200a levels in the 12 paired NSCLC and adjacent normal tissue samples were analyzed by RT-qPCR. (B) The mean values of miR-200a expression in the 12 paired NSCLC and adjacent normal tissue samples were calculated. *P<0.05 vs. Normal. (C) The miR-200a levels in a normal lung cell line (HEL299) and two NSCLC cell lines (H1703 and H2172) were analyzed by RT-qPCR. *P<0.05 vs. HEL299. The data are presented as the mean ± standard deviation. Each experiment was repeated at least three times. RT-qPCR, reverse transcription-quantitative polymerase chain reaction; miR-200a, microRNA-200a; NSCLC, non-small cell lung cancer.

Statistical analysis. Data are presented as the mean ± standard deviation from at least three independent experiments. A two-tailed Student's t-test was used to analyze the mean value between two groups. The difference between groups was analyzed using one-way analysis of variance with post hoc analysis by a Student-Newman-Keuls test, when three or more groups were compared. Correlation analysis was performed using two-tailed Pearson's correlation coefficient analysis. P<0.05 was considered to indicate a statistically significant difference. All analysis was performed using SPSS software (version 16.0; SPSS, Inc.).

Results

miR-200a levels in lung cancer tissues. Initially, the current study measured miR-200a levels in 12 paired NSCLC and adjacent normal tissue samples by RT-qPCR. In all cases, the tumor tissue exhibited lower miR-200a levels compared with the matched-adjacent normal tissue (Fig. 1A). The mean values of miR-200a in the 12 paired NSCLC and adjacent normal tissue samples were calculated. Tumor tissue demonstrated a significantly lower mean level of miR-200a compared with healthy tissue (Fig. 1B). Similarly, a subsequent comparison revealed that the level of miR-200a was significantly lower in two NSCLC cell lines, H1703 and H2172, compared with a normal lung cell line, HEL299 (Fig. 1C).

In vitro role of miR-200a in NSCLC cell lines. As H1703 and H2172 cells demonstrated significantly lower levels of miR-200a, the current study regulated the miR-200a levels by transfection with miR-200a mimic and miR-200a ASO, which upregulate and downregulate miR-200a, respectively. As expected, transfection with the miR-200a mimic significantly increased the level of miR-200a in H1703 and H2172 cells, whereas miR-200a ASO transfection significantly decreased the level of miR-200a (Fig. 2A). Following transfection, cell growth was assessed. Upregulation of miR-200a inhibited cell growth, while downregulation of miR-200a significantly increased cell growth in H1703 and H2172 cells (Fig. 2B). Cell apoptosis was also investigated and it was identified that transfection with miR-200a mimic

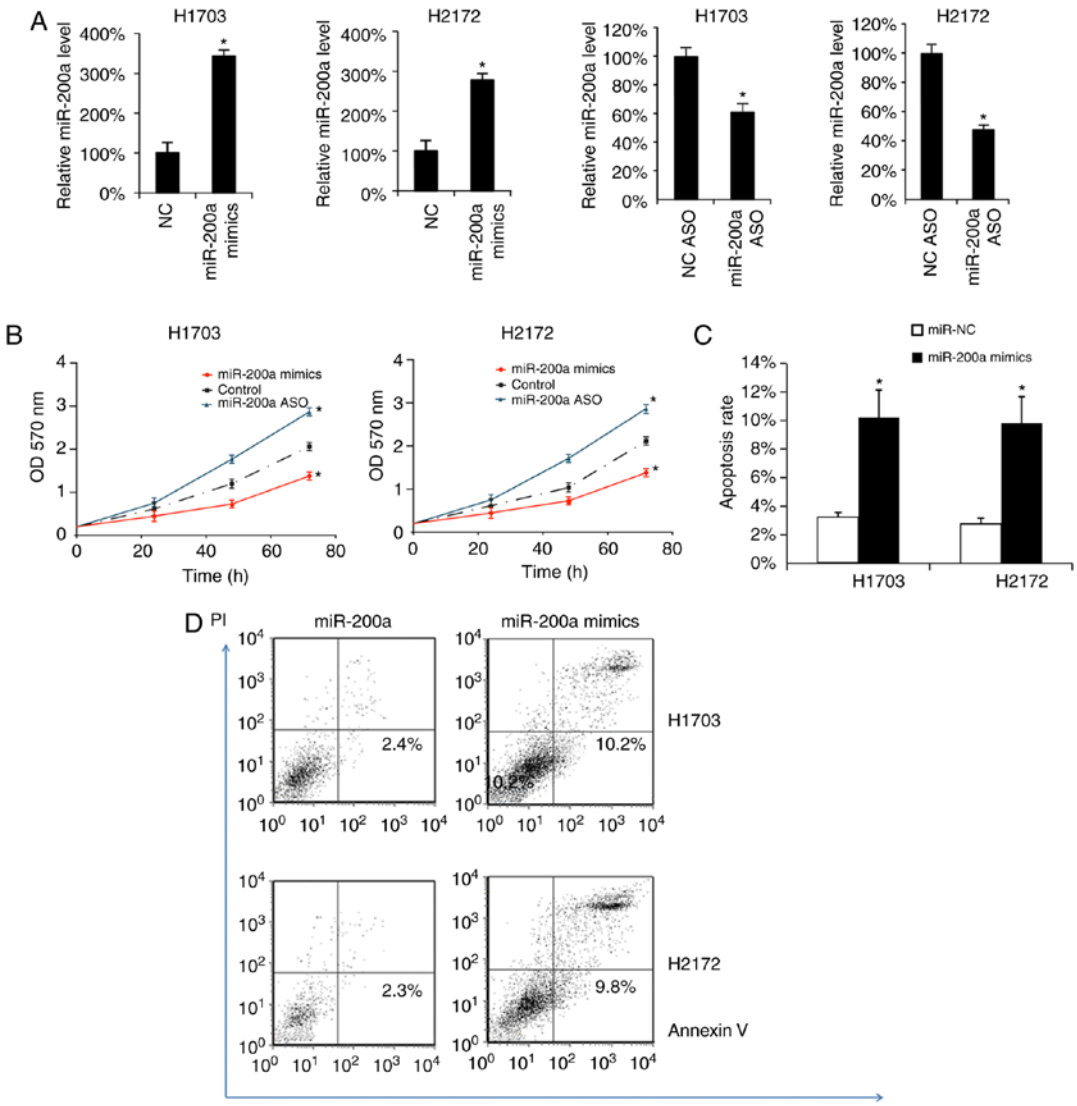

Figure 2. Overexpression of miR-200a inhibits H1703 and H2172 cell proliferation and promotes cells apoptosis. H1703 and H2172 cells were separately transfected with miR-200a mimic, NC, miR-200a ASO or NC ASO. (A) At 24 h transfection miR-200a expression was evaluated by reverse transcription-quantitative polymerase chain reaction. *P<0.05 vs. NC or NC ASO. (B) Following transfection with miR-200a mimic and miR-200a ASO, the cellular proliferation of H1703 and H2172 was assessed by an MTT assay. *P<0.05 vs. control. (C) At 48 h after transfection with miR-200a mimic, H1703 and H2172 cells were stained with Annexin V-FITC and PI, and the cell apoptosis rate was evaluated by flow cytometry. (D) The histogram of cell apoptosis rate. *P<0.05 vs. miR-NC. miR-200a, microRNA-200a; NC, negative control; ASO, antisense oligonucleotide; OD, optical density; FITC, fluorescein isothiocyanate; PI, propidium iodide.

significantly increased the apoptosis rate in H1703 and H2172 cells (Fig. 2C and D).

RHPN2 is a target gene of miR-200a. RHPN2 encodes a member of the rhophilin family of Ras-homologous (Rho)-GTPase binding proteins and may be involved in the organization of the actin cytoskeleton (29). In malignant glioma, RHPN2 drives mesenchymal transformation (30). The current study identified that RHPN2 was a target gene of miR-200a. The binding sites of miR-200a in the 3'UTR of RHPN2 and the mutant 3'UTR of RHPN2 are presented in Fig. 3A. The two 3'UTRs were cloned into a luciferase reporter plasmid and a control plasmid. A miR-200a mimic and the reporter plasmid or control plasmid were co-transfected into H1703 cells. The activity of luciferase was assessed 12 h following transfection. The upregulation of miR-200a inhibited luciferase activity of the wild-type 3'UTR of RHPN2 but not the mutated version, indicating that miR-200a targets RHPN2 in H1703 cells (Fig. 3B). RHPN2 protein levels following transfection with the miR-200a mimic were determined. The transfection inhibited the RHPN2 protein levels in H1703 cells (Fig. 3C).

RHPN2 levels in NSCLC tissues. The protein level of RHPN2 was analyzed in the 12 paired NSCLC and adjacent normal tissue samples by IHC. The NSCLC tissue samples consistently demonstrated higher protein levels of RHPN2 compared with adjacent normal tissue. Representative paired NSCLC and adjacent normal tissue sample images are presented in Fig. 4A. The protein levels of RHPN2 were quantified using a point scoring system. All 12 NSCLC tissue samples scored a higher mean RHPN2 level compared with the adjacent normal tissue (Fig. 4B). Next, the RHPN2 mRNA levels were analyzed in the 12 paired NSCLC and adjacent normal tissue samples. Higher mean RHPN2 mRNA levels were consistently

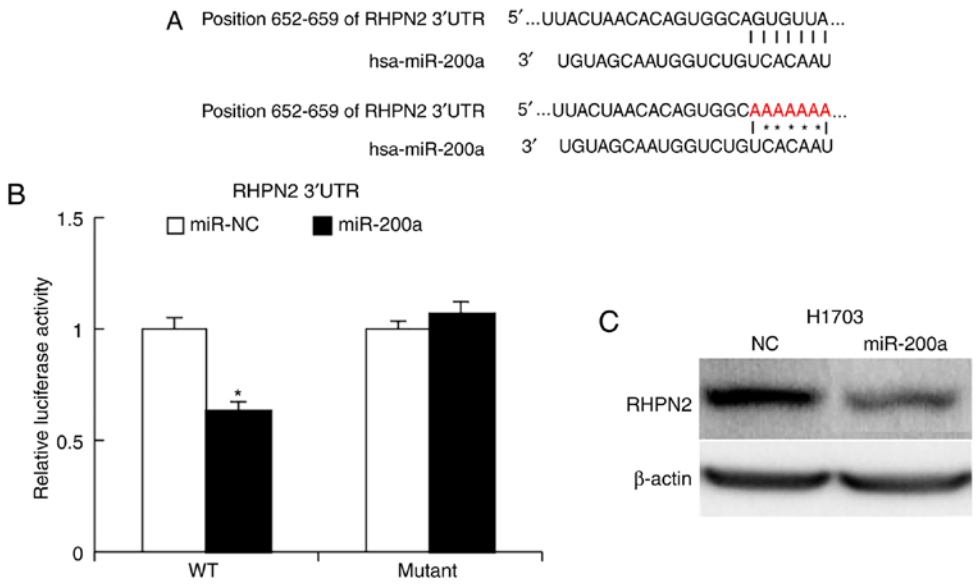

Figure 3. RHPN2 was identified as a target gene of miR-200a in H1703 cells. (A) The miR-200a binding site and its mutated version in the 3'UTR of RHPN2. (B) miR-200a mimic and a reporter plasmid containing the wild-type or mutated 3'UTR sequence of RHPN2 were transfected into H1703 cells and 48 h later the luciferase activity was analyzed. *P<0.05 vs. miR-NC. (C) miR-200a mimic was transfected into H1703 cells and the abundance of RHPN2 protein was determined by western blotting. Each experiment was repeated at least three times. RHPN2, Rhophilin Rho GTPase binding protein 2; miR-200a, microRNA-200a; 3'UTR, 3' untranslated region; NC, negative control; WT, wild-type.

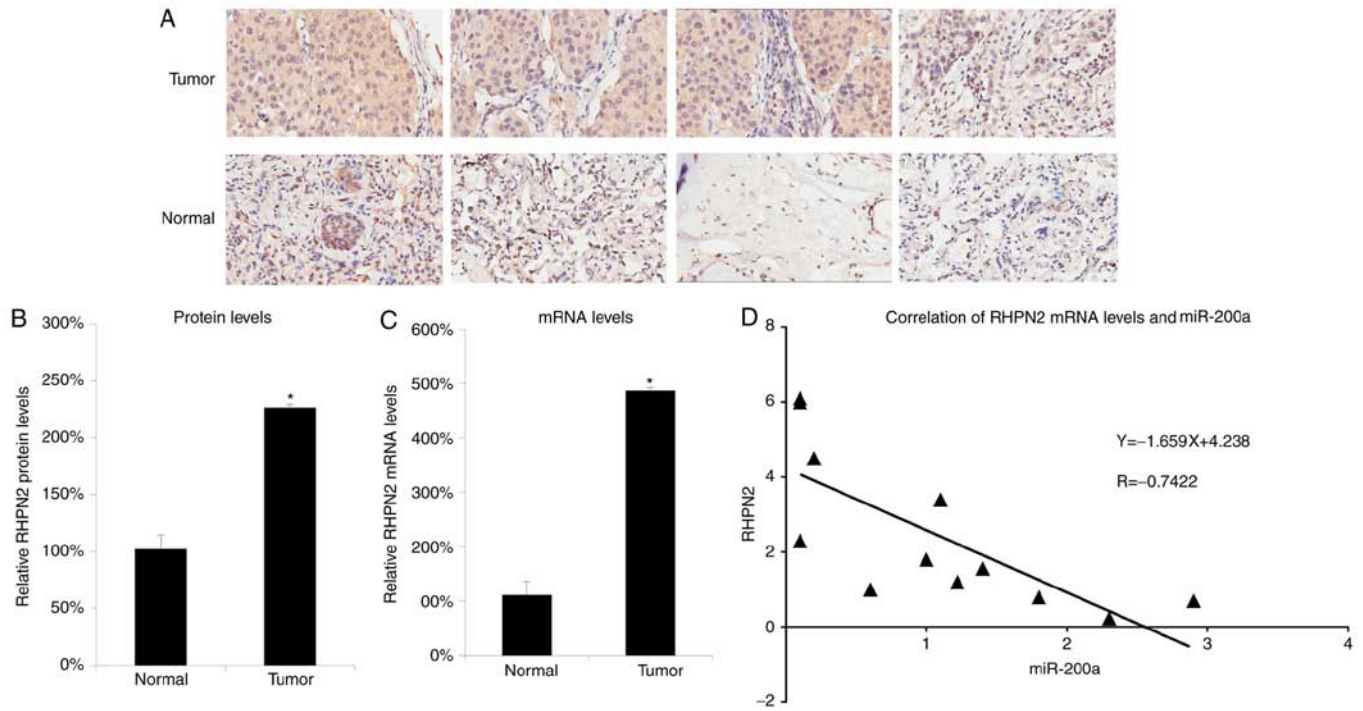

Figure 4. RHPN2 protein and mRNA levels in NSCLC tissue samples. A total of 12 paired NSCLC and adjacent normal tissue samples were collected for analysis of RHPN2 protein and mRNA levels. Tumor tissue samples and matched-adjacent normal tissue samples were processed by IHC for analysis of RHPN2 protein expression. (A) Representative figures of tumor and matched-adjacent normal tissues are presented. Magnification, x200. (B) IHC staining was scored according to the following criteria: - (0-10% of the nucleated cells stained); + (10-40% stained); ++ (40-70% stained) and +++ (70-100% stained). The mean expression of RHPN2 in NSCLC tissue samples was calculated relative to the mean expression of RHPN2 in normal tissue samples, which was arbitrarily defined as 100%. (C) The RHPN2 mRNA levels in the 12 paired NSCLC and adjacent normal tissue samples were analyzed by RT-qPCR. The mean expression of RHPN2 mRNA in normal tissue samples was arbitrarily defined as 100%. (D) RHPN2 mRNA and miR-200a levels in the 12 NSCLC tissue samples were analyzed by RT-qPCR. P<0.05. Correlation analysis was performed using two-tailed Pearson's correlation coefficient analysis. Each experiment was repeated at least three times. *P<0.05 vs. Normal. RHPN2, Rhophilin Rho GTPase binding protein 2; mRNA, messenger RNA; miR-200a, microRNA-200a; RT-qPCR, reverse transcription-quantitative polymerase chain reaction; NSCLC, non-small cell lung carcinoma; IHC, immunohistochemistry.

identified in the 12 NSCLC tissue samples compared with the matched tissue samples (Fig. 4C). In addition, RHPN2 mRNA expression levels were assayed in the 12 NSCLC tissue samples and correlation analysis was performed between

RHPN2 mRNA expression and miR-200a expression. This analysis revealed a negative correlation between RHPN2 mRNA expression levels and miR-200a expression levels in the 12 NSCLC tissue samples (Fig. 4D).

Discussion

The role of miR-200a in NSCLC was investigated in the current study. NSCLC tissue samples demonstrated lower levels of miR-200a compared with matched normal lung tissues. *In vitro* experiments revealed that miR-200a inhibited growth of two NSCLC cell lines and promoted cell apoptosis. Furthermore, RHPN2 was identified as a target gene of miR-200a.

The miR-200 family are regulators of epithelial-mesenchymal transition (EMT) (31). E-cadherin repressors, zinc finger E-box binding homeobox (ZEB)1 and ZEB2, are target genes of the miR-200 family, serving a role in the EMT process (15). The current study revealed that miR-200a could target RHPN2. Previously, to the best of our knowledge, the function of RHPN2 in cancer has only been assessed in malignant glioma, in which it promotes mesenchymal transformation (30). The current study revealed that higher RHPN2 protein levels are expressed in NSCLC tissues compared with matched normal tissues. Furthermore, it was demonstrated that miR-200a could target RHPN2. The current data may be explained by two hypotheses. Firstly, RHPN2 may be a key factor in the EMT. Secondly, RHPN2 may participate in the EMT by regulating ZEB1 and ZEB2. The current study suggests miR-200a plays a role in the EMT process via RHPN2.

miR-200a, miR-141 and miR-429 belong to the miR-200 family (32). Previous studies have identified that miR-141 and miR-429 promote the proliferation of NSCLC cells (33,34). However, the current study revealed that miR-200a inhibited growth of two NSCLC cell lines and promoted cell apoptosis. miR-141 regulates PH domain leucine-rich-repeats protein phosphatase 1 (PHLPP1) and PHLPP2, and miR-492 regulates deleted in liver cancer 1 (DLC-1), a tumor suppressor in NSCLC (34). Therefore, it can be proposed that miR-200a regulates RHPN2.

The current study only collected 12 paired NSCLC and adjacent normal tissue samples. Regardless, the results suggested that miR-200a serves an anti-tumor role in NSCLC via the targeting of RHPN2. Lower levels of miR-200a in NSCLC tissues compared with healthy tissues may be associated with the survival rate of patients with NSCLC. Future studies may investigate the prognostic value of miR-200a in NSCLC.

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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

LZ designed the study. YH and TB collected patient data and performed the cell experiments. ZL and GJ performed the molecular biology experiments. LZ wrote the manuscript. All authors read and approved the final manuscript.

Ethics approval and consent to participate

The present study was approved by The Ethics Committee of Sichuan University and all patients provided written informed consent.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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