MiR-381 enhances the sensitivity of non-small cell lung cancer to radiotherapy by targeting ROCK2 to regulate NF-κB signaling pathway

Jiuning Huang
Tianjin Medical University Cancer Institute and Hospital

Jing Zeng
Tianjin Medical University Cancer Institute and Hospital

Shuping Zhang
Yantai Affiliated Hospital of Binzhou Medical University

Jundong Cai
Tianjin Medical University Cancer Institute and Hospital

Wulong Wang
Tianjin Medical University Cancer Institute and Hospital

Kaikai Zhao
Yantai Affiliated Hospital of Binzhou Medical University

Xuelei Wang
Yantai Affiliated Hospital of Binzhou Medical University

Qingsong Pang
Tianjin Medical University Cancer Institute and Hospital

Ping Wang (pingwang2020@163.com)
Tianjin Medical University Cancer Institute and Hospital

Research article

Keywords: miR-381, non-small cell lung cancer (NSCLC), radiotherapy, ROCK2, NF-κB signaling pathway, sensitivity

DOI: https://doi.org/10.21203/rs.3.rs-26677/v1

License: This work is licensed under a Creative Commons Attribution 4.0 International License.
Read Full License
Abstract

Background

We investigated the effect of miR-381 on the sensitivity of non-small cell lung cancer (NSCLC) to radiotherapy, and examined its possible mechanism.

Methods

NSCLC A549 cells and miR-381 overexpression and gene silencing cell lines were treated with radiotherapy. The cell proliferation was tested by CCK-8 assay and colony formation. Flow cytometry and TUNEL were used to detect the cell apoptosis. The expression of nuclear factor kappa B (NF-κB) signaling pathway related proteins were detected by western blot. NF-κB signaling pathway activator and inhibitor cell lines were further constructed and the above experiments were repeated. Double luciferase assay was used to verify the target of miR-381. Furthermore, a nude mouse xenograft model was constructed and treated with radiotherapy. The tumor volume and tumor weight were measured. The expression of PCNA protein in tumor tissues was observed by immunohistochemistry. The apoptosis related proteins in tumor tissues were detected by western blot.

Results

The mRNA expression of miR-381 was increased after radiotherapy treatment. Radiotherapy treatment also can inhibit the proliferation and promote apoptosis of A549 cells. Compared with radiotherapy group, cell proliferation was significantly decreased and apoptosis was significantly increased in miR-381 overexpression group (p < 0.05). Moreover, ROCK2 is a target of miR-381, and overexpression of miR-381 can down-regulate the expression of ROCK2 protein. In nude mice, miR-381 mimic interference can reduce cell tumorigenicity and proliferation, and increase the apoptosis. However, the indicators above were contrary in miR-381 silencing group. Verication experiments further verify that NF-κB signaling pathway activator can reverse the role of miR-381.

Conclusion

MiR-381 overexpression could enhance the sensitivity of NSCLC to radiotherapy by targeting ROCK2 to inhibit NF-κB signaling pathway.

Introduction

At present, there are 1.2 million new cases of lung cancer every year and one person dies from lung cancer every 30 seconds worldwide.\textsuperscript{1,2} Lung cancer is clinically divided into small cell lung cancer (SCLC) and non-small cell lung cancer (NSCLC) by histological methods. Non-small cell lung cancer accounts for about 85% of all lung cancer.\textsuperscript{3} Additionally, there are differences between males and females in the incidence of lung cancer, smoking prevalence and life expectancy.\textsuperscript{4,5} Only a small percentage of NSCLC
patients are diagnosed at an early stage, when the tumor can be treated by surgical resection.\textsuperscript{2} About 75\% of NSCLC patients present with a locally advanced or metastatic disease at the time of diagnosis.\textsuperscript{2} Despite treatment advances, conventional chemotherapy and radiation therapy are still the main treatments for patients with lung cancer.

MicroRNAs (miRNAs) is a type of non-coding RNA with a size of about 22 nt, which has a wide range of biological functions such as proliferation, invasion, metastasis and cell survival.\textsuperscript{6-8} MiRNAs can specifically bind to mRNA to recruit related RNases and cause mRNA degradation, thereby blocking the expression of protein-encoding genes and affecting its biological function.\textsuperscript{9} Numerous studies have demonstrated that miRNAs involve in the occurrence, development, invasion, and metastasis of various human malignant tumors.\textsuperscript{10,11} Moreover, growing amount of evidences have suggested that miRNAs have significant differential expression in lung cancer patients with different prognosis and benign lung lesions.\textsuperscript{12} One of those miRNAs, miR-381 was shown to serve as a tumor suppressor in some cancers.\textsuperscript{13,14} Rho-associated protein kinase 2 (ROCK2), a member of the serine/threonine AGC kinase family, is a regulator that can regulate cell proliferation, invasion and metastasis.\textsuperscript{15} Study has reported that ROCK2 may be could affect cancer treatment outcomes.\textsuperscript{16} Furthermore, Yilin Xie et al. found that miR-381 could serve as a tumor suppressor by targeting ROCK2.\textsuperscript{17} Radiotherapy is the main treatment for most cancers. Improving the sensitivity of cancer cells to radiotherapy has an important role for the treatment of various malignant cancers. Therefore, it is significant to study the effects and potential underlying mechanisms of miR-381 on the sensitivity of NSCLC to radiotherapy.

In this study, we hypothesize that miR-381 overexpression could enhance the sensitivity of NSCLC to radiotherapy, and further sought to verify whether miR-381 improves the sensitivity of NSCLC to radiotherapy through regulating nuclear factor kappa B (NF-κB) signaling pathway.

**Materials And Methods**

**Cell culture**

Human non-small cell lung cancer cells A549 were purchased from Shanghai Institute of Cell Biology, Chinese Academy of Sciences (Shanghai, China). Cells were cultured in RPMI-1640 medium (GIBCO, Invitrogen, Grand Island, NY, USA) containing 10\% fetal bovine serum (Solarbio, Beijing, China) and 1\% penicillin-streptomycin (Solarbio, Beijing, China) at 37°C, 5\% CO\textsubscript{2}.

**Cell transfection and grouping**

Cells were passaged and inoculated on a 6-well plate. When the cells reached 70\% confluence, transfection was performed according to the lentiviral transfection instruction (Shanghai Jikai Biotechnology Co., Ltd., Shanghai, China). The cells were divided into 6 groups: i) blank control group (BC), no treatment; ii) radiotherapy group (Rad), cells were exposed to a single dose of X-rays using a linear accelerator (RadSource, Suwanee, GA, USA) at a dose rate of 1.15 Gy/min and 160 kv X-ray
energy,\textsuperscript{18} no transfection; iii) miR-381 overexpression negative control group (NC-1), cells were radiotherapy treatment and transfected with miR-381 scramble; iv) miR-381 overexpression group (miR-381), cells were radiotherapy treatment and transfected with miR-381 mimics; v) miR-381 silencing negative control group (NC-2), cells were radiotherapy treatment and transfected with miR-381 inhibitor negative control; and vi) miR-381 silencing group (si-miR), cells were radiotherapy treatment and transfected with miR-381 inhibitor.

**Cell counting kit (CCK)-8 assay**

The proliferation ability of cells was performed by the CCK-8 assay kit (Dojindo, Japan) according to the manufacturer’s protocol. Logarithmic growth phase cells were cultured in 96-well plates at a density of $2 \times 10^4$ cells/ml, 100 µl per well. After culture for 24 h, 48 h, 72 h, and 96 h, 10µl of CCK-8 solution was added into plates and then followed with incubation for 4 h at 37°C, 5% CO$_2$. The optical density (OD) was measured at 450 nm.

**Colony formation assay**

Logarithmic growth phase cells were digested with 0.25% trypsin and adjusted to 250 cells/ml. 2 ml/well cells were cultured in a 6-well plate at 37°C, 5% CO$_2$ for 2-3 weeks and the fresh medium was changed every 3 days. The cells were fixed in methanol and each well was added with 1mL Ji Giemsa working fluid and stained for 30 min. After washed twice with ultrapure water, the record was imaged by a camera.

**Flow cytometry**

24 h after transfection, the cells were collected and resuspended with pre-chilled 1× PBS, and centrifuged at 1000 rpm for 5-10 min. After washing, 300 µl 1× binding buffer was added to the cells for suspension, and 5 µl Annexin V-FITC was added. The cells were mixed and incubated in dark for 15min. Then, 5 µl propidium iodide (PI) was added and incubated in the dark for 5 min. 200 µl 1× binding buffer was added prior to analysis using flow cytometry (Beckman Coulter, Brea, CA, USA). CellQuest software (BD Bioscences, San Diego, USA) was used to analyze the results.

**TdT-mediated dUTP nick end labeling (TUNEL) staining**

TUNEL apoptosis detection kit (C1098, Beyotime, Nanjing, China) was used to analyze the cells apoptosis. Cells were crawled and fixed with 4% paraformaldehyde for 30 min. After cultured in PBS containing 0.3% Triton X-100 at room temperature for 5 min, 0.3% H$_2$O$_2$ methanol solution was added to the cells and deactivated the samples for 20 min. TUNEL reaction mixed droplets were prepared and added to the specimen, reacted in a dark humid chamber at 37°C for 1 h. After washed once with PBS, 0.3 ml labeled reaction stop solution dropwise were added to the cells and incubated at room temperature for 10 min. Then, the cells were incubated with 100 µl Streptavidin-HRP working solution at room temperature for 30 min. The cells were developed with DAB, then followed by hematoxylin counterstained, ethanol gradient dehydrated, xylene transparent, and neutral resin sealed. The apoptotic cells were
observed under a ×400 optical microscope (BX50, Olympus, Japan) and counted by Aperio Imagescope 11.1 software. The cells were tan or brownish yellow and had morphological characteristics of apoptotic cells were judged as apoptotic positive cells. Apoptotic index (AI) = (the number of apoptotic positive cells ÷ the number of total cells) × 100%.

**Quantitative real-time polymerase chain reaction (qRT-PCR)**

The cells were collected and centrifuged at 4°C, 12,000 rpm for 15 min. Total RNA was extracted with TRizol kit (Takara, Dalian, China) (OD260/OD280 between 1.8-2.0, indicating that the RNA purity is acceptable). The cDNA was synthesized by the reverse transcription kit (Applied Biosystems, Waltham, MA, USA). Mastercycler® nexus X2 (Eppendorf, Hamburg, Germany) was used for RT-PCR. The data was processed by the \(2^{-\Delta\Delta Ct}\) method, and the relative expression level was calculated using U6 mRNA as an internal reference. The sequence of the primers (Shanghai Sheng gong Bioengineering Technology Service Co., Ltd., Shanghai, China) are as follows: miR-381, Forward: 5’-GTCTATACAAGGGCAAGCTCTC-3’ and Reverse: 5’-ATCCATGACAGATCCCTACCG-3’. U6, Forward: 5’-GACCTCTATGCAACACAGT-3’, Reverse: 5’-AGTACTTGCCTCAGGAGGA-3’.

**Western blot**

The cells were lysed, and centrifugated at 2000rpm for 20min. The protein concentration was measured by BCA kit (Solarbio, Beijing, China). 40 μg samples of each group were separated by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (Mini-protean-3, Bio-Rad, Hercules, CA, USA), and transferred to polyvinylidene difluoride (PVDF) membrane (Merck, Darmstadt, Germany). After blocking with 5% skimmed milk, the membranes were incubated overnight at 4˚C with primary rabbit anti-human antibodies against ROCK2 (1:1000, ab71589, Abcam), P65 (1:500, orb229138, Biorbyt, Cambridge, UK), p-P65 (1:500, orb304662, Biorbyt, Cambridge, UK), IkBa (1:500, orb223182, Biorbyt, Cambridge, UK), p-IkBα (1:500, orb223035, Biorbyt, Cambridge, UK), and β-actin (1:2000, orb178392, Biorbyt, Cambridge, UK). Subsequently, horseradish peroxidase-conjugated goat anti-rabbit immunoglobulin G secondary antibody (1:1000, #7074, Cell Signaling Technology, USA) was used. The membranes were observed and recorded by enhanced chemiluminescence (ECL) system (ImageQuant LAS 4000, General Electric Company, Fairfield, CT, USA). Protein expression levels were normalized with β-actin, scanned and quantified by Image J (NIH) software.

**Verification**

To verify the relationship between miR-381 and NF-κB signaling pathway, the cells were divided into 5 groups: i) blank control group (BC), no treatment; ii) radiotherapy group (Rad), cells were radiotherapy treatment and no transfection; iii) miR-381 mimic group (miR-381), cells were radiotherapy treatment and transfected with miR-381 mimics; iv) NF-κB inhibitor (PDTC, American Sigma) group (PDTC), cells were radiotherapy treatment and cultured with 100 μmol/L PDTC reagent; and v) miR-381 simulant + NF-κB activator (PMA, Sigma, USA) group (miR + PMA), cells were radiotherapy treatment and transfected with miR-381 mimics cultured with 100 μg/L PMA reagent. The above experiments were repeated.
Animals and ethics statement

36 female Balb/c nude mice (4 weeks old, 16-18 g weight) were purchased from Jinan Pengyue Experimental Animal Breeding Co., Ltd., license number SCXK (Lu) 2014-0007. The feeding environment were maintained in the standard conditions of 26-28°C temperature, 50-60% relative humidity, 12 h light/dark cycle with the freely accessed to drinking water and food. Animal experiments were conducted following the National Institute of Health (NIH) guidelines (NIH Pub. No. 85-23, revised 1996). The experiments have been reviewed and approved by the Animal Protection and Use Committee of Tianjin Medical University Cancer Institute and Hospital.

Construction and grouping of lung cancer xenograft model

The lung cancer cells A549 in logarithmic growth phase were digested with 0.25% trypsin, collected and counted. The cell concentration was adjusted to $2.5 \times 10^6$ cells/ ml, and 0.2 ml was inoculated into the soft skin of the right forelimb back of the nude mouse. 36 mice were randomly divided into 6 group (n=6): i) blank control group (BC); ii) radiotherapy group (Rad); iii) miR-381 mimic group (miR-381); iv) Radiotherapy + miR-381 overexpression negative control group (NC-1); v) Radiotherapy + miR-381 silent group (si-miR), and vi) Radiotherapy + miR-381 silent negative control group (NC-2). One week later, except for the control group, the other nude mice were irradiated with 6MV X-rays at a dose rate of 2 Gy/min. A lead plate with a distance of 100 cm and the latter 3 mm covered other parts of the nude mice, and the dose was 10 Gy.

Tumor volume calculation

The tumor's long diameter (L) and short diameter (W) were measured every week with vernier caliper. The tumor volume (V) = ($L \times W^2$)/2. Tumor was measured on a weekly basis for 4 weeks and the tumor growth curve was drawn. 4 weeks later, nude mice were anesthetized by intraperitoneal injection of 0.6% sodium pentobarbital (40 mg/kg, New Asiatic Pharmaceutical, China) and sacrificed by cervical dislocation.

Immunohistochemistry

After conventional sectioning of the tumor tissue, the slices were baked, dewaxed with xylene, and sequentially hydrated with a gradient ethanol solution. 3% $\text{H}_2\text{O}_2$ methanol solution was used to inactivate processing for 20 min. The slices were heated with citrate buffer (pH 6.0) for 10 min and sealed with 5% BSA for 20 min. After blocking with 5% goat serum (Gibco, USA) for 20 min, the slices were incubated with rabbit anti-human PCNA (1: 500, orb251877, Biorbyt, Cambridge, UK) polyclonal antibody overnight at 4 °C. Then, the goat anti-rabbit IgG (1: 1000, ABIN101988, antibodies-online, Germany) labeled with horseradish peroxidase was used for secondary antibody incubation. 3,3’-Diaminobenzidine staining, hematoxylin counterstaining, dehydration and sealed. The expression of PCNA in each group was observed under a × 400 light microscope (Olympus, Japan) and count using AperioImagescope 11.1 software.
**Western blot**

Tumor tissue was ground, homogenized, and centrifuged at 10,000 rpm for 10 min. The primary antibodies were rabbit anti-human Bax (1:500, orb224426, Biorbyt, Cambridge, UK), Bcl-2 (1:500, orb228150, Biorbyt, Cambridge, UK), and Caspase-3 (1:500, orb10231, Biorbyt, Cambridge, UK). Other experimental steps were the same as above western blot.

**Double luciferase reporter assay**

The wild type and mutant 3’UTRs of ROCK2 were amplified in pGL3/luciferase vector (Promega, Madison, WI, USA) and cloned to the downstream of the luciferase gene. The cells were tested for luciferase activity using a dual luciferase reporter system (Promega) 48 h after transfection according to the instructions.

**Statistical methods**

SPSS19.0 statistical software was used to analyze the data. The results were expressed as mean ± standard deviation (SD). Data analysis between the two groups using t test. Means of analysis between multiple groups were analyzed by single factor analysis of variance (ANOVA), and subsequent analysis was performed by LSD test. P < 0.05 indicates that the difference is statistically significant.

**Results**

**Effects of radiotherapy treatment on the proliferation and apoptosis of A549 cells**

As shown in Figure 1, compared with BC group, radiotherapy treatment significantly inhibited the proliferation and promoted apoptosis of A549 cells (p < 0.05). Meanwhile, the mRNA expression of miR-381 in Rad group was remarkably higher than the BC group (p < 0.05).

**Effects of miR-381 on the proliferation of A549 cells**

Contrasted with Rad group, the level of miR-381 mRNA in miR-381 group was remarkably raised, and that was remarkably declined in si-miR group (p < 0.05, Figure 2A). Contrasted with Rad group, the cell proliferation ability of miR-381 group was remarkably reduced, and the cell proliferation ability of si-miR group was significantly increased (p < 0.05, Figure 2B and 2C). Moreover, the cell proliferation ability of si-miR group was significantly higher than the miR-381 group (p < 0.05). The results revealed that miR-381 overexpression could enhance the sensitivity of NSCLC to radiotherapy.

**Effects of miR-381 on the apoptosis of A549 cells and NF-κB signaling pathway**

As shown in Figure 3, contrasted with Rad group, the apoptotic rate and apoptosis index of miR-381 group were remarkably raised, and the expression of p-P65/P65 was remarkably declined, moreover, the expression of p-IκBα/IκBα was remarkably raised (p < 0.05). However, the results of the si-miR group were exactly opposite. Contrasted with miR-381 group, the apoptosis rate and apoptosis index of si-miR group were significantly reduced, and the expression of p-P65/P65 was significantly increased, moreover, the
expression of p-IκBα/IκBα was significantly reduced (p < 0.05). Those results suggested that miR-381 overexpression could enhance the sensitivity of NSCLC to radiotherapy and inhibit NF-κB signaling pathway.

**miR-381 regulates the proliferation of A549 cells by regulating NF-κB signaling pathway**

To verify the relationship between miR-381 and NF-κB signaling pathway activator and inhibitor cell lines were further constructed. Figure 4 shown that compared with BC group, the cell proliferation ability of other groups was significantly reduced (p < 0.05). Simultaneously, compared with Rad group, the cell proliferation ability of miR-381 and PDTC groups was dramatically decreased (p < 0.05). However, compared with miR-381 and PDTC groups, the cell proliferation ability of miR + PMA group was significantly increased (p < 0.05). Those results indicated that miR-381 overexpression enhances the sensitivity of NSCLC to radiotherapy by inhibiting NF-κB signaling pathway.

**miR-381 regulates the apoptosis of A549 cells by regulating NF-κB signaling pathway**

Furthermore, as exhibited in Figures 5, compared with BC group, the cell apoptosis rate and apoptosis index of other groups were significantly increased, and the expression of p-P65/P65 was significantly reduced, moreover, the expression of p-IκBα/IκBα was significantly increased (p < 0.05). Compared with Rad group, the apoptotic rate and apoptosis index of miR-381 and PDTC groups were significantly increased, the expression of p-P65/P65 in the NF-κB signaling pathway was dramatically decreased, and the expression of p-IκBα/IκBα was significantly increased (p < 0.05). However, the results of the miR + PMA group were exactly opposite. All those results verified that miR-381 overexpression enhances the sensitivity of NSCLC to radiotherapy by inhibiting NF-κB signaling pathway.

**Effects of miR-381 on NSCLC xenografts**

A nude mouse xenograft model was further constructed and treated with radiotherapy (Figure 6). Compared with BC group, the tumor growth rate in the other groups was significantly slowed, tumor volume, weight, and the percentage of PCNA-positive cells were significantly decreased, while, the expression of apoptosis-related proteins were significantly increased (p < 0.05). Compared with Rad group, the miR-381 group has a better tumor suppressive effect, but the si-miR group had the opposite effect (p < 0.05). Compared with miR-381 group, the tumor growth rate in the si-miR group was significantly accelerated, the tumor volume, weight, and the percentage of PCNA-positive cells were significantly increased, and the expressions of Bax and Caspase-9 were significantly decreased (p < 0.05). All these findings demonstrated that miR-381 overexpression could inhibit cell tumorigenicity and proliferation, and promote the apoptosis.

**ROCK2 is the target of miR-381**

As shown in Figure 7, a bioinformatics search identified ROCK2 is the target of miR-381. A dual luciferase reporting system was used to further verify whether miR-381 targets ROCK2. The results showed that miR-381 reduced the luciferase activity of ROCK containing WT 3'UTR, but did not decrease the luciferase
activity of ROCK containing Mut 3'UTR. Compared with the BC group, the ROCK protein expression was significantly increased in radiotherapy group (p < 0.05). Moreover, miR-381 overexpression could reduce the expression of ROCK2 and miR-381 silencing could increase the expression of ROCK2.

**Discussions**

As a member of miRNA family, miR-381 affects metastasis in some type of cancers.\(^{14-17}\) In this study, the potential role of miR-381 in lung cancer was firstly evaluated. We found that the mRNA expression of miR-381 in radiotherapy cells was remarkably higher than that in blank control cells, and the miR-381 overexpression treatment could inhibit proliferation and promote apoptosis of NSCLC. Those results indicated that miR-381 plays a positive role in the treatment of NSCLC. Furthermore, through predicted by databases and dual luciferase assay, we found that ROCK2 is the target of miR-381. The protein expression of the ROCK2 were significantly down-regulated after miR-381 mimic transfection and miR-381 silencing could increase the expression of ROCK2.

Cell proliferation is the foundation of organism growth, development, reproduction, and heredity. The cancer progression usually associated with abnormal cells proliferation, migration and invasion. Uncontrolled proliferation provides a survival advantage of cancer cells to resist conventional chemotherapeutic agents.\(^{19-21}\) In this study, CCK-8 and cloning formation assays confirmed the miR-381 overexpression could inhibit cell tumorigenicity and proliferation, which suggested that miR-381 overexpression could enhance the sensitivity of NSCLC to radiotherapy. Apoptosis is a basic biological phenomenon of cells and plays a necessary role in the removal of unwanted or abnormal cells by multicellular organisms. Apoptosis is the main cause of tumor cell death and is the core mechanism for preventing tumor growth.\(^{22}\) Flow cytometry and TUNEL staining showed miR-381 overexpression increased the apoptotic level of NSCLC cells. The expression of Bax and Caspase-9 were further evaluated, and the apoptosis related proteins in tumor tissues were increased after miR-381 mimic interference.

The mechanisms involved in the NSCLC have not yet been completely elucidated. A number of studies found that NF-κB signaling pathway plays highly important roles in cell proliferation, apoptosis, angiogenesis, inflammation, metastasis, and drug resistance.\(^{23-25}\) A variety of stimuli coalesce on NF-κB activation, which can in turn mediate varied transcriptional programs.\(^{26}\) In this study, the protein expression of P65, IκBα, and their phosphorylated were detected by western blot. The results found that after silencing miR-381, the expression of p-P65/P65 was increased and the expression of p-IκBα/IκBα was reduced, which indicated that miR-381 overexpression could inhibit NF-κB signaling pathway. To further define whether miR-381 overexpression enhances the sensitivity of NSCLC to radiotherapy through the NF-κB signaling pathway, the pathway activator and inhibitor were used. The results showed that the addition of NF-κB inhibitor evidently decreased the proliferation ability and increased cell apoptosis. All those results verified that miR-381 overexpression enhances the sensitivity of NSCLC to radiotherapy by inhibiting NF-κB signaling pathway.
Conclusion

In sum, miR-381 overexpression could inhibit cell tumorigenicity and proliferation, and promote the apoptosis, which suggested that miR-381 overexpression could enhance the sensitivity of NSCLC to radiotherapy. The possible mechanism is related to targeting ROCK2 to suppress NF-κB signaling pathway. Therefore, miR-381 maybe a potential therapeutic agent for further treatment of lung cancer.

Declarations

All manuscripts must contain the following sections under the heading 'Declarations':

Ethics approval and consent to participate
Animal experiments were followed the NIH guidelines (NIH Pub. No. 85-23, revised 1996) and have been approved by the Animal Protection and Use Committee of Tianjin Medical University Cancer Institute and Hospital.

Consent for publication
Not applicable

Availability of data and materials
The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

Competing interests
The authors declare that they have no competing interests

Funding
Not applicable

Authors’ contributions
JH, JZ and SZ developed the model, carried out the parameter estimations and planned as well as performed the mass transfer experiments. JH also wrote the main part of the manuscript and took part in the planning and execution of the fermentation experiments. JH, JC and WW took part in the development of the model, planned and carried out the main part of the fermentation experiments, analyzed the results and assisted in the mass transfer experiments. KZ and XW also wrote parts of the manuscript. QP and PW participated in the coordination of the study and reviewed the manuscript. All authors read and approved the final manuscript..

Acknowledgements
Not applicable
References

1. Mayekar MK, Bivona TG. Current Landscape of Targeted Therapy in Lung Cancer. Clin Pharmacol Ther. 2017;102(5):757-764.

2. Osmani L, Askin F, Gabrielson E, Li QK. Current WHO guidelines and the critical role of immunohistochemical markers in the subclassification of non-small cell lung carcinoma (NSCLC): Moving from targeted therapy to immunotherapy. Semin Cancer Biol. 2018;52(Pt 1):103-109.

3. Oser MG, Niederst MJ, Sequist LV, Engelman JA. Transformation from non-small-cell lung cancer to small-cell lung cancer: molecular drivers and cells of origin. Lancet Oncol. 2015;16(4):e165-e172.

4. Torre LA, Bray F, Siegel RL, Ferlay J, Lortet-Tieulent J, Jemal A. Global cancer statistics, 2012. CA Cancer J Clin. 2015;65(2):87-108.

5. Liutkute V, Veryga A, Štelemekas M, Goštautaite Midttun N. Burden of smoking in Lithuania: attributable mortality and years of potential life lost. Eur J Public Health. 2017;27(4):736-741.

6. Liu B, Li J, Cairns MJ. Identifying miRNAs, targets and functions. Brief Bioinform. 2014;15(1):1-19.

7. Nair VS, Maeda LS, Ioannidis JP. Clinical outcome prediction by microRNAs in human cancer: a systematic review. J Natl Cancer Inst. 2012;104(7):528-540.

8. Maskey N, Li D, Xu H, Song H, Wu C, Hua K, Song J, Fang L. MicroRNA-340 inhibits invasion and metastasis by downregulating ROCK1 in breast cancer cells. Oncol Lett. 2017;14(2):2261-2267.

9. Theil K, Imami K, Rajewsky N. Identification of proteins and miRNAs that specifically bind an mRNA in vivo. Nat Commun. 2019;10(1):4205.

10. Rupaimoole R, Slack FJ. MicroRNA therapeutics: towards a new era for the management of cancer and other diseases. Nat Rev Drug Discov. 2017;16(3):203-222.

11. Di Leva G, Garofalo M, Croce CM. MicroRNAs in cancer. Annu Rev Pathol. 2014;9:287-314.

12. Pastuszak-Lewandoska D, Kordiak J, Czarnecka KH, et al. Expression analysis of three miRNAs, miR-26a, miR-29b and miR-519d, in relation to MMP-2 expression level in non-small cell lung cancer patients: a pilot study. Med Oncol. 2016;33(8):96.

13. Tzeng HE, Chang AC, Tsai CH, Wang SW, Tang CH. Basic fibroblast growth factor promotes VEGF-C-dependent lymphangiogenesis via inhibition of miR-381 in human chondrosarcoma cells. 2016;7(25):38566-38578.

14. Li Y, Zhao C, Yu Z, et al. Low expression of miR-381 is a favorable prognosis factor and enhances the chemosensitivity of osteosarcoma. 2016;7(42):68585-68596.

15. Kümpfer S, Mardakheh FK, McCarthy A, et al. Rho-associated kinase (ROCK) function is essential for cell cycle progression, senescence and tumorigenesis. Elife. 2016;5:e12994.

16. Ebata T, Mitsui Y, Sugimoto W, et al. Substrate Stiffness Influences Doxorubicin-Induced p53 Activation via ROCK2 Expression. Biomed Res Int. 2017;2017:5158961.

17. Xie Y, Qi J, Zhu C, Zhao D, Liao G. MiR-381 functions as a tumor suppressor in gastric cancer by targeting ROCK2. Int J Clin Exp Pathol. 2019;12(1):164-172.
18. Tang Y, Cui Y, Li Z, et al. Radiation-induced miR-208a increases the proliferation and radioresistance by targeting p21 in human lung cancer cells. J Exp Clin Cancer Res. 2016;35:20.

19. Iciek MB, Kowalczyk-Pachel D, Kwiecień I, Dudek MB. Effects of different garlic-derived allyl sulfides on peroxidative processes and anaerobic sulfur metabolism in mouse liver. Phytother Res. 2012;26(3):425-431.

20. Yue X, Zhang Z, Liang X, et al. Zinc fingers and homeoboxes 2 inhibits hepatocellular carcinoma cell proliferation and represses expression of Cyclins A and E. Gastroenterology. 2012;142(7):1559-70.e2.

21. Zhao D, Zhang T, Hou XM, Ling XL. Knockdown of fascin-1 expression suppresses cell migration and invasion of non-small cell lung cancer by regulating the MAPK pathway. Biochem Biophys Res Commun. 2018;497(2):694-699.

22. Tian X, Wang Y, Li S, Yue W, Tian H. ZHX2 inhibits proliferation and promotes apoptosis of human lung cancer cells through targeting p38MAPK pathway. Cancer Biomark. 2020;27(1):75-84.

23. de Castro Barbosa ML, da Conceicao RA, Fraga AGM, et al. NF-κB Signaling Pathway Inhibitors as Anticancer Drug Candidates. Anticancer Agents Med Chem. 2017;17(4):483-490.

24. Soleimani A, Rahmani F, Ferns GA, Ryzhikov M, Avan A, Hassanian SM. Role of the NF-κB signaling pathway in the pathogenesis of colorectal cancer. Gene. 2020;726:144132.

25. Hesari A, Ghasemi F, Salarinia R, et al. Effects of curcumin on NF-κB, AP-1, and Wnt/β-catenin signaling pathway in hepatitis B virus infection. J Cell Biochem. 2018;119(10):7898-7904.

26. Oeckinghaus A, Hayden MS, Ghosh S. Crosstalk in NF-κB signaling pathways. Nat Immunol. 2011;12(8):695-708.

Figures
Figure 1

Effects of radiotherapy treatment on the proliferation and apoptosis of A549 cells. (A) CCK-8 assay and (B) cloning formation (×400) were used to detect cell proliferation; (C) Flow cytometry and (D) TUNEL staining (×400) were used to detect apoptosis; (E) qRT-PCR was used to detect the mRNA expression of miR-381. *p < 0.05 compared with BC group.
Figure 2

Effects of miR-381 on the proliferation of A549 cells. (A) qRT-PCR was used to detect the mRNA expression of miR-381; (B) CCK-8 assay and (C) cloning formation (×400) were used to detect cell proliferation. *p < 0.05 compared with BC group; #p < 0.05 compared with Rad group; ^p < 0.05 compared with miR-381 group.
Figure 3

Effects of miR-381 on the apoptosis of A549 cells and NF-κB signaling pathway. (A) Flow cytometry and (B) TUNEL staining (×400) were used to detect apoptosis; (C) Western blot was used to detect the expression of NF-κB signaling pathway related proteins. *p < 0.05 compared with BC group; #p < 0.05 compared with Rad group; ^p < 0.05 compared with miR-381 group.
miR-381 regulates the proliferation of A549 cells by regulating NF-κB signaling pathway. (A) Flow cytometry and (B) TUNEL staining (×400) were used to detect apoptosis. *p < 0.05 compared with BC group; #p < 0.05 compared with Rad group; ^p < 0.05 compared with miR-381 group; &p < 0.05 compared with PDTC group.
Figure 5

miR-381 regulates the apoptosis of A549 cells by regulating NF-κB signaling pathway. (A) Flow cytometry and (B) TUNEL staining (×400) were used to detect apoptosis; (C) Western blot was used to detect the expression of NF-κB signaling pathway related proteins. *p < 0.05 compared with BC group; #p < 0.05 compared with Rad group; ^p < 0.05 compared with miR-381 group; &p < 0.05 compared with PDTC group.
Figure 6

Effects of miR-381 on NSCLC xenografts. (A) Tumor volume; (B) Tumor photograph; (C) Tumor weight; (D) Immunohistochemistry was used to detect the expression of PCNA; (E) Western blot was used to detect the expression of apoptosis related proteins. *p < 0.05 compared with BC group; #p < 0.05 compared with Rad group; ^p < 0.05 compared with miR-381 group.
Figure 7

ROCK2 is a target of miR-381. (A) predicted binding site of miR-381 and ROCK2 3'UTR region; (B) double luciferase report; (C) Western blot was used to detect the expression of ROCK2 protein. *p < 0.05 compared with BC group; #p < 0.05 compared with Rad group; ^p < 0.05 compared with miR-381 group.