

MicroRNA-520a-3p inhibits cell growth and metastasis of non-small cell lung cancer through PI3K/AKT/mTOR signaling pathway

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Abstract. – **OBJECTIVE:** MicroRNAs are a class of small non-coding RNAs that be involved in the pathogenesis of non-small cell lung cancer (NSCLC). The purpose of this study was to evaluate the effects of miR-520a-3p in cell growth and metastasis.

MATERIALS AND METHODS: The mimics and inhibitor of miR-520a-3p were used to identify the effects of miR-520a-3p on cell proliferation and apoptosis using methylthiazol tetrazolium (MTT) assay and flow-cytometric method, respectively. Transwell assay was used to evaluate the cell migration and invasion. The protein expression levels related PI3K/AKT/mTOR signaling pathway were measured by Western blot.

RESULTS: The results showed that miR-520a-3p overexpression could significantly inhibit cell proliferation and induce apoptosis, suppress cell migration and invasion. miR-520a-3p overexpression could markedly reduce the ratio of p-AKT/AKT, p-PI3K/PI3K and Bcl-2/Bax, the levels of mTOR, matrix metalloproteinase (MMP-2) and matrix metalloproteinase-9 (MMP-9) compared with control. However, miR-520a-3p overexpression could increase caspase-3 expression compared with control group. After inhibited the expression of miR-520a-3p, the capacity of cell proliferation, migration and invasion were increased, cell apoptosis was inhibited compared with control group. The ratio of p-AKT/AKT, p-PI3K/PI3K and Bcl-2/Bax, the levels of mTOR, MMP-2 and MMP-9 were increased compared with control group.

CONCLUSION: Our study suggested that miR-520a-3p could suppress the NSCLC proliferation, migration and invasion through PI3K/AKT/mTOR signaling pathway.

Keywords:

miR-520a-3p, Non-small cell lung cancer, PI3K/AKT/mTOR, Apoptosis, MMP2, MMP9.

Abbreviations

AKT, protein kinase B; ANOVA, analysis of variance; Bax, Bcl-2 Associated X protein; Bcl-2, B-cell lymphoma/leukemia-2; DMSO, dimethyl sulfoxide; ECL,

enhanced chemiluminescence; HRP, horseradish peroxidase; NSCLC, non-small cell lung cancer; UTR, Untranslated region; MMP, matrix metalloproteinases; mTOR, mammalian target of rapamycin; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; PBS, phosphate buffered saline; FBS, fetal bovine serum; FITC, fluorescein isothiocyanate; 4EBP1, eukaryotic initiation factor 4E; PBS, phosphate-buffered saline; PI, propidium iodide; PI3K, phosphoinositide 3-kinase; PVDF, polyvinylidene difluoride; qRT-PCR, quantitative Real-time polymerase chain reaction; S, standard deviation; RIPA, radio immunoprecipitation assay; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; SYBR, synergy blue.

Introduction

Lung cancer is considered as one of the most serious diseases worldwide¹. The incidence of lung cancer increases by the 16% around the world in the two decades^{2,3}. Non-small cell lung cancer (NSCLC) is the predominant type, accounting for 80% of all lung cancers. During these years, signs of progress have been made for treatment of lung cancer, though the molecular mechanisms need to be elucidated⁴⁻⁶.

MicroRNAs are a family of small, single-stranded, endogenous and non-coding RNAs, around 18-25 nucleotides. They could suppress gene expression after post-transcription through binding to 3'-untranslated regions (UTR) of target gene mRNA⁷⁻⁹. Previous studies have demonstrated that miRNA plays an important role in tumors and it is involved in the gene regulation, cell differentiation, proliferation, apoptosis and metastasis. miR-520a-3p has been identified as an oncogene or tumor suppressor, such as hepatocellular carcinoma, pancreatic cancer and so on^{10,11}. However, the underlying mechanism of miR-520a-3p on NSCLC metastasis needs to be clarified.

Phosphoinositide 3-kinase/protein kinaseB/Mammalian target of rapamycin (PI3K/AKT/mTOR) is the key pathway involved in cell cycle,

proliferation, and energy metabolism. It is one of the most mutated pathways in tumor^{12,13}. PI3K is a kinase located on the cellular membrane, stimulated by several hormones. Once PI3K is activated, it can phosphorylate the downstream kinases such as AKT. Also, this pathway plays an important role on cellular metabolism and protein synthesis. Dysregulation of this pathway is participated in several cancers, targeting of this pathway is considered as a potential therapeutic strategy of NSCLC¹⁴.

Matrix metalloproteinases (MMPs) are zinc-dependent secreted proteinases, which play an important role in invasion, apoptosis in tumor progression, especially in relation to tumor metastasis. NSCLC cell line NCI-H1975 was used in this study. Several studies have been used to explore the role of miR-520a-3p in lung cancer. The mRNA expression level of miR-520a-3p was measured with qRT-PCR. MTT assay and flow-cytometry were used to evaluate the cell proliferation and apoptosis, respectively. Cell migration and invasion were detected by the transwell assay. The proteins related to PI3K/ATK/mTOR were measured with Western blot.

Materials and Methods

Cell Culture and Transfection

NSCLC cell line NCI-H1975 was obtained from ATCC (Manassas, VA, USA). Cells were cultured in the high glucose Dulbecco's Modified Eagle Medium (DMEM) (Gibco, Rockville, MD, USA) with 1% fetal bovine serum (FBS) (Gibco, Rockville, MD, USA), and kept at 37°C in a 5% CO₂ incubator. Cells were transfected with mimics 50 nM (Ambion, Foster City, CA, USA) and inhibitor 55 nM (Dharmacon, Lafayette, CO, USA) of miR-520a-3p with Lipofectamine 2000 for 24 h following the instruction.

qRT-PCR Assay

Quantitative real-time PCR assay was applied to evaluate the expression level of miRNA of NCI-H1975 cells. For RNA isolation, total RNA was extracted with TRIzol reagent (Gibco, Rockville, MD, USA), the concentration and purity of RNA were determined at 260/280 nm using a nanodrop spectrophotometer (ND-2000, Thermo Fisher Scientific, Waltham, MA, USA). 1 µg of each extracted RNA was used to cDNA synthesis, and RT primer was 5'-CTCAACTGGTGTCTGGAGTC-GGCAATTCAGTTGAGACAGTCCAAA-3'. RT cDNA synthesis was conducted in 14 µl reaction

mixture, containing 1 µl reverse transcriptase (50 U) and 1 µl oligo (dT) primer (Bio-Rad, Hercules, CA, USA), according to manufacturer's instructions (TaKaRa, Otsu, Shiga, Japan). The cDNA was used as a template; the relative expression levels of miR-520a-3p and U6 from rats receiving experimental treatment were determined by PCR. The sequence of the primers for qRT-PCR was as follows: miR-520a-3p, Forward, 5'-ACACGAGCAGCTGGG AAAGTGCTTCC-3' and Reverse, 5'-CTCAACTGGTGTCTGGT-3'. Each 20 µl reaction system contained 2 µl of cDNA, 1 µl SYBR Premix Ex Taq (TaKaRa, Otsu, Shiga, Japan), 10 µM of both sense and antisense primers. All data for each sample were measured in triplicate and using 2^{-ΔΔCt} method.

Cell Proliferation Assay

The proliferation of NCI-H1975 cells was detected by cell proliferation reagent kit I methylthiazolyl tetrazolium (MTT) (Sigma-Aldrich, St. Louis, MO, USA). Cells treated with mimics, inhibitor and vehicle were seeded into a 96-well plate at 4×10³ and incubated for 24 h. Each well was added 200 µl MTT solution (Aladdin, Shanghai, China) (5 mg/ml) and incubated for 4 h at 37°C. Dimethyl sulfoxide (DMSO) 150 µl/well was added for 5 min before reading at 490 nm with VersaMax microplate reader (Molecular Devices, Sunnyvale, CA, USA).

Flow-Cytometric Analysis of Apoptosis

The FITC Annexin V/PI apoptosis detection kit (BD Biosciences, San Diego, CA, USA) was used to measure cell apoptosis. The NCI-H1975 cells were treated with mimics, inhibitor and vehicle for 24 h in 6-well plate. Cells were washed twice with cold phosphate buffered saline (PBS) and harvested trypsinization, and re-suspended in binding buffer. The cells were double stained with annexin V-fluorescein isothiocyanate (FITC) and propidium iodide (PI) (J&K Scientific Ltd., Beijing, China) for 15 min at 37°C. Then, cells were detected with flow cytometer (CyAn ADP9, Beckman Coulter, CA, USA). All cells were divided into living cells, early apoptotic cells and late apoptotic cells. The percentage of cells with apoptotic nuclei (% apoptosis) was calculated.

Cell Migration and Invasion Assays

For the migration assay, cells were placed in the upper chamber of an insert (8 µm pore size; Costar, Switzerland) with 200 µl serum-free medium. In the lower chamber, 500 µl medium with

10% FBS were added. The cells were incubated at 37°C for 12 h in incubator. Cells on the filter surface were fixed with 4% formaldehyde (Beyotime Biotechnology, Shanghai, China), stained with 0.1% crystal violet solution (Beyotime Biotechnology, Shanghai, China) for 30 min.

For the invasion assay, the upper chamber of transwell insert was coated with Matrigel Matrix (BD Biosciences, Franklin Lakes, NJ, USA). Cells were transfected with mimics, inhibitor and vehicle for 48 h. The cells were harvested and seeded into the upper chamber of the insert with serum-free medium for 24 h. The cells that remained on the upper membrane were removed with cotton wool. The invaded cells were stained with 0.1% crystal violet solution for 30 min after fixed with 4% formaldehyde. Images were captured with light microscope (Olympus, Tokyo, Japan) and cells numbers were calculated on five fields of each insert.

Western Blot Assay

The protein samples from each group were resolved by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE). The protein was extracted by lysing cells in radioimmuno precipitation assay (RIPA) buffer containing protease and phosphatase inhibitor cocktail. Protein concentrations were determined using bicinchoninic acid (BCA) Protein Assay reagent kit (Thermo Fisher Scientific, Waltham, MA, USA). Equal amount of protein (40 µg) were separated by SDS-PAGE, and were then transferred to polyvinylidene difluoride (PVDF) membranes (Millipore, Billerica, MA, USA). The nonspecific binding of antibodies was blocked with 5% non-fat dried milk in PBS and then incubated with the primary antibodies PI3K, 1:500, p-PI3K, 1:500, Akt, 1:500, p-Akt, 1:500 (Santa Cruz Biotechno-

logy, Santa Cruz, CA, USA), mTOR, 1:500 (Abcam, Cambridge, MA, USA), Bcl-2 1:500, Bax, 1:500, caspase-3, 1:500 (Santa Cruz Biotechnology, Santa Cruz, CA, USA), MMP-2, 1:2000, MMP-9, 1:2000, and β -actin, 1:1000 (Abcam, Cambridge, MA, USA) were used followed by the application of the secondary antibodies consisting of horseradish-peroxidase (HRP) conjugated goat anti-rabbit/mouse IgG 1:1000 (Santa Cruz Biotechnology, Santa Cruz, CA, USA). The protein bands were detected by enhanced chemiluminescence (ECL) reagent. The images were analyzed with Quantity One molecular imaging system (Bio-Rad, Hercules, CA, USA).

Statistical Analysis

Data are expressed as mean $\pm$ standard deviation (SD). Statistical differences were evaluated by software SPSS 19.0 (SPSS IBM, Armonk, NY, USA). Statistical analysis was performed using one-way analysis of variance (ANOVA) followed by LSD test. $p < 0.05$ was considered as statistically significant.

Results

miRNA-520a-3p Suppresses Cell Proliferation and Induces Apoptosis

MTT assay was used to evaluate the effects of miRNA-520a-3p on NSCLC cell line NCI-H1975 on cell proliferation. The results showed that mimics treatment could significantly suppress the cell proliferation compared with vehicle (Figure 1A) over 96 h after transfection. When cells were transfected with inhibitor of miRNA-520a-3p, the viable cells were markedly increased compared with vehicle (Figure 1A).

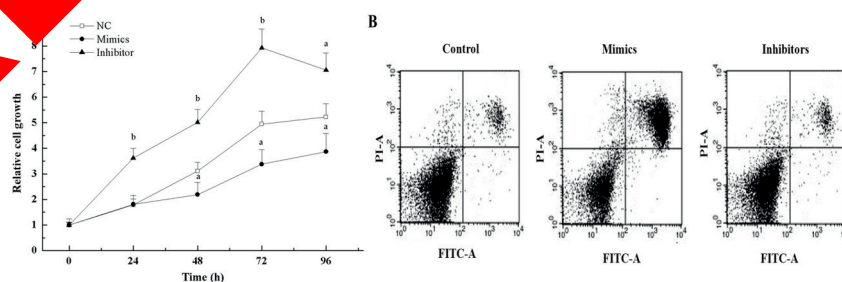


Figure 1. Effect of miRNA-520a-3p on cell proliferation and apoptosis of NCI-H1975 cell. **A**, MTT assay was performed to evaluate the proliferation at different time points; $p < 0.05$, compared with control. **B**, Apoptosis rates were determined by flow cytometry. Data represent the mean $\pm$ SD; $p < 0.01$ compared with control.

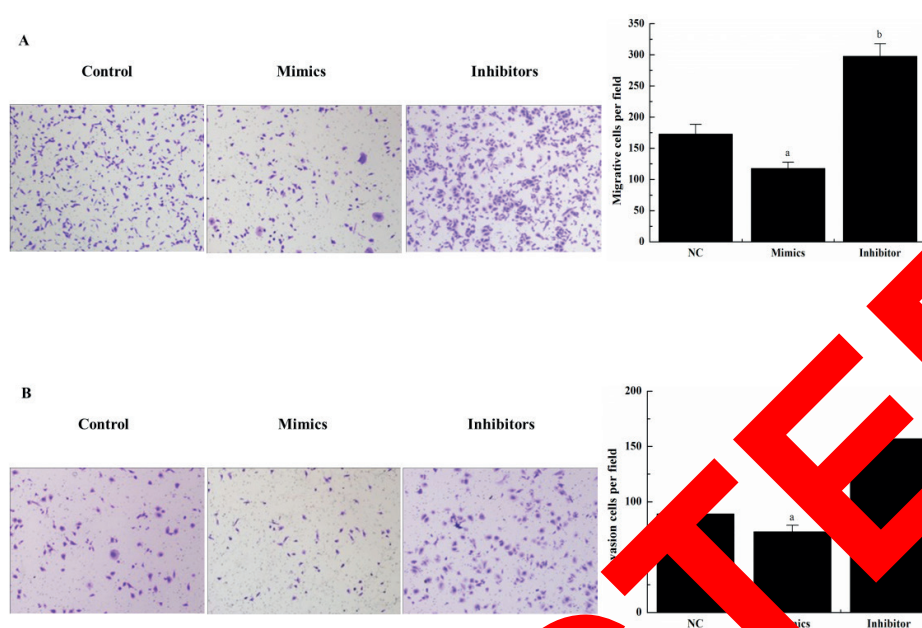


Figure 2. Effect of miRNA-520a-3p on cell migration and invasion of NCI-H1975 cell. **A**, Effect of miRNA-520a-3p on cell migration of NCI-H1975 cell. $p < 0.05$ compared with control. **B**, Effect of miRNA-520a-3p on cell invasion of NCI-H1975 cell. Data represent the mean $\pm$ SD. $p < 0.01$ compared with control.

The flow cytometry was used to detect the effect of miRNA-520a-3p on cell apoptosis. The results demonstrated that mimics treatment could increase cell apoptosis compared with control group, while the inhibitor treatment had no significant difference compared with control group (Figure 1B). These results suggested that miRNA-520a-3p could suppress NSCLC proliferation and induce apoptosis.

miRNA-520p-3a Inhibits Cell Migration and Invasion

Cell migration and invasion are a key aspect of cancer metastasis. Transwell assay is widely used to measure these two abilities. As shown in our study, mimics treatment could significantly inhibit migration and invasion (Figure 2A, $p < 0.05$), while inhibitor could significantly up-regulate these abilities compared with control group (Figure 2B, $p < 0.01$). The results demonstrated miRNA-520p-3a may have a tumor suppressor function.

miRNA-520p-3a Affects PI3K/AKT/mTOR Pathway

PI3K/AKT/mTOR pathway is important in cellular metabolism and protein synthesis. To determine the activation of PI3K/AKT/mTOR pathway in NSCLC cell, cells were transfected with mimics and inhibitor of miRNA-520p-3a; the phosphoryla-

tion of these proteins was evaluated by Western blot. As shown in Figure 3, the phosphorylation of PI3K (Figure 3A, $p < 0.01$) and AKT (Figure 3B, $p < 0.05$) was significantly reduced after mimics' treatment. When cells treated with inhibitor of miRNA-520p-3a, the phosphorylation of PI3K (Figure 3A, $p < 0.01$) and AKT (Figure 3B, $p < 0.05$) was markedly promoted. The mimics could markedly block the protein expression of mTOR (Figure 3C, $p < 0.05$), meanwhile the cells transfected with inhibitor could induce the expression of mTOR (Figure 3C, $p < 0.05$).

Caspase-3, Bcl-2 and Bax play a key role in cell apoptosis and tumorigenesis. Mimics of miRNA-520a-3p treatment could reduce expression of Bcl-2, and increase Bax expression (Figure 3D, $p < 0.01$) compared to control group. While the expression of caspase-3 was markedly increased in mimics group compared with control group (Figure 3E, $p < 0.05$).

MMP2 and MMP9 are critical factors involved in tumor cell migration and invasion. Compared with control group, the protein levels were significantly lower in the mimics treated group (Figure 3F, $p < 0.01$), while inhibitor could markedly increase MMP2 and MMP9 levels (Figure 3F, $p < 0.01$). These results indicated that miRNA-520a-3p may affect tumor cell apoptosis through PI3K/AKT/mTOR pathway.

Discussion

NSCLC is one of the most serious diseases worldwide, and the underlying mechanism is very complex. There is growing evidence that miRNA plays a key role in progression of NSCLC tumor cells¹⁶⁻¹⁹. Previous studies demonstrated that miRNA deregulation participated in the initiation, progression and metastasis of many cancers through different signaling pathway, which regulates cell proliferation, differentiation, apoptosis, migration and invasion. Moreover, some studies found that some miRNAs play a crucial role in oncogenes or tumors. Several non-coding RNAs were identified as NSCLC metastasis and as hallmarks of cancers. In the present study, miRNA-520a-3p, a typical multifunction miRNA, was evaluated in NSCLC cell line NCI-H1975^{20,21}. Our results demonstrated that miRNA-520a-3p could suppress tumor cell proliferation, induce apoptosis and hinder cancer cell migration and invasions. Yu et al²² reported that increased expression of miRNA-520a-3p markedly inhibited NSCLC proliferation, promoted NSCLC apoptosis and down-regulated cell invasion and metastasis capacities. Our data were consistent with Yu's study. To explore the molecular mechanism

of miRNA -520a-3p in NSCLC cell line, the PI3K/AKT/mTOR pathway related proteins were detected in this study. PI3K/AKT/mTOR pathway plays an important role of mediator of many biological processes such as cell proliferation, migration, cell cycle progression and apoptosis^{15,23}. PI3K/AKT/mTOR signaling pathway is involved in many human cancers. PI3K, a lipid kinase consisting as a heterodimer consisting of regulatory subunit p85 and a catalytic subunit p110, is activated by many cytokines in extracellular matrix through auto-phosphorylation, and this involvement in cell growth and survival. AKT is the important molecule downstream to PI3K. Over-phosphorylated AKT was closely related to chemoresistance in NSCLC cell lines²⁴⁻²⁶. The AKT over-phosphorylated, could activate downstream targets in cytoplasm and nucleus to promote cell growth and survival. mTOR is the key substrate of AKT. Activated mTOR could stimulate eukaryotic initiation factor 4E (4EBP1) and result in cell proliferation. Dysregulation of this pathway could induce cell apoptosis and alter metastasis. In this study, miRNA-520a-3p could significantly reduce the phosphorylation of PI3K and AKT, downregulating the protein level of

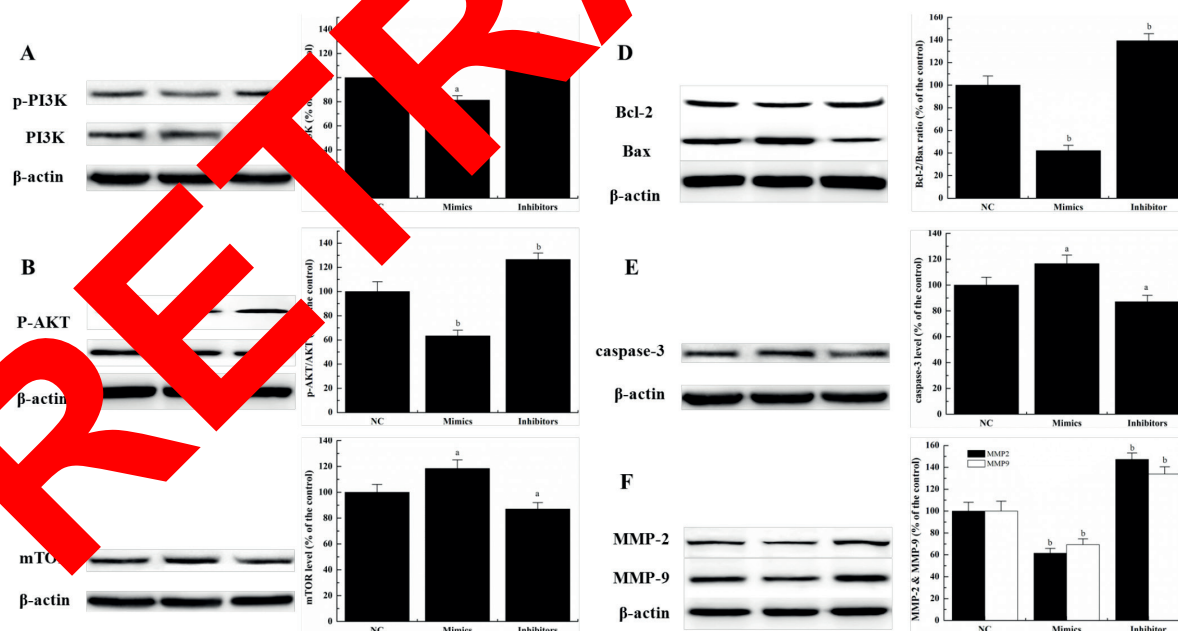


Figure 3. The effects of miRNA-520a-3p on related protein expression with PI3K/AKT/mTOR pathway in NCI-H1975 cell line. **A**, Western blot analysis of p-PI3K and PI3K; $p < 0.05$, compared with control. **B**, Western blot analysis of p-AKT and AKT; $p < 0.01$, compared with control. **C**, Western blot analysis of mTOR. **D**, Western blot analysis of Bcl-2 and Bax. **E**, Western blot analysis of caspase-3. **F**, Western blot analysis of MMP-2 and MMP-9. Data represent the mean $\pm$ SD.

that miRNA-520a-3p is a very promising inhibitor of PI3K/AKT/mTOR pathway and could significantly inhibit cell proliferation and induce cell apoptosis. Apoptosis is one of most important physiological cell processes that control cell death. Caspase-3, Bcl-2 and Bax play a key role in cell apoptosis and tumorigenesis. Caspase-3 is the important protein of caspase enzyme family, which is involved in promoting cell apoptosis²⁷. Most cell apoptosis-inducing factors eventually cause cell apoptosis through caspase pathway. Bcl-2 and Bax are the two main group proteins of Bcl-2 family. Bcl-2 is the vital anti-apoptosis protein, whereas Bax is pro-apoptosis protein. Bax could release cytochrome c from mitochondria where activates caspase-3, which could form a heterodimer with Bcl-2. These proteins were also measured in this experiment. When cells were treated with miRNA -520a-3p mimics, the ratio of Bcl-2/Bax was attenuated, and the expression of caspase-3 was increased. These findings were also consistent with previous results. MMPs are considered to play a role in cell proliferation, adhesion, migration, and metastasis^{28,29}. MMPs could reduce the expression of type IV collagen since it is an important component of extracellular matrix. Down-regulation of MMP2 and MMP9 could inhibit NSCLC migration, invasion and metastasis. We detected the expression of the MMP-2 and MMP-9. The results indicated that miRNA-520a-3p treated with mimics could reduce MMP2 and MMP9 expression in NCI-H1975 cells. Mao et al³⁰ also reported that siRNA-TMEM98 treatment for 48 h inhibited NSCLC cells (A549 and H460) invasion and migration through down-regulated MMP2 and MMP9 expression. These studies could help understanding the pathogenesis, progression, and potential treatment of NSCLC and facilitate the diagnostic and therapeutic approaches in this disease.

Conclusions

Our results demonstrated that miRNA-520a-3p could suppress cell proliferation, apoptosis and metastasis of NSCLC NCI-H1975 cell through PI3K/AKT/mTOR pathway. miRNA-520a-3p could be considered as a potential target of therapeutic NSCLC in clinic study.

Conflict of Interest

The Authors declare that they have no conflict of interest.

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