Original Paper

Effect of miR-21 on Apoptosis in Lung Cancer Cell Through Inhibiting the PI3K/Akt/NF- κ B Signaling Pathway *in Vitro* and *in Vivo*

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Key Words

Mir-21 • ASPP2 • NSCLC • PI3K/Akt • NF- κ B

Abstract

Background/Aims: Lung cancer is one of the most common malignancies in the world. Apoptosis-stimulating protein of p53 (ASPP2), a tumorigenesis related protein, plays a critical role in the initiation and development of various types of cancers. However, the effect of ASPP2 on lung cancer remains unknown. The purpose of this study aims to investigate the mechanism of ASPP2 regulated by miR-21 in lung cancer *in vitro* and *in vivo*. **Methods:** In the study, migration and invasion assays, apoptosis assay, caspase activity assay, TUNEL staining, real time PCR and western blot were used to investigate the mechanism of ASPP2 regulated by miR-21 in lung cancer *in vitro* and *in vivo*. **Results:** We demonstrated that the miR-21 inhibitor induced apoptosis through inhibiting the PI3K/Akt/NF- κ B signaling pathway in non-small cell lung carcinoma (NSCLC). Moreover, ASPP2 was directly targeted by miR-21 in NSCLC cells. Down-regulation of miR-21 suppressed cell migration and invasion, as well as the EMT signaling pathway in NSCLC cells. Furthermore, the miR-21 inhibitor induced cell apoptosis via the caspase dependent pathway in NSCLC cells. The miR-21 inhibitor enhanced caspase-3, 8, 9 activity in NSCLC cells. In addition, the caspase inhibitor significantly reduced the apoptosis induced by the miR-21 inhibitor in NSCLC cells. **Conclusions:** Our results revealed that the miR-21 inhibitor could induce apoptosis through inhibiting the PI3K/Akt/NF- κ B signaling pathway in human NSCLC cells, and might serve as a therapeutic strategy to treat NSCLC.

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Published by S. Karger AG, Basel

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Introduction

Lung cancer, which occurs in the bronchiolar epithelium, is the most life-threatening neoplasm and one of the main problems that needs to be solved in the world [1-3]. Additionally, the incidence of lung cancer has already ranked first in male malignancies [4, 5]. Apoptosis is the process of an autonomously and orderly death of cells controlled by genes, and it plays a major role in the maintenance of the internal stability of cells [6-8]. Excessive apoptosis can cause atrophic diseases, and deficiency of apoptosis can cause the loss of cell proliferation, which leads to carcinogenesis [9-11]. Previous research has already demonstrated that the apoptosis-stimulating protein of p53 (ASPP2) is closely related to tumorigenesis [12, 13]. EGFR can reduce ASPP2-induced apoptosis by the activation of the PI3K/Akt signaling pathway in hepatoma cells [14]. In colorectal cancer cells, ASPP2 induces apoptosis by binding and activating the RAS/PI3K/AKT signaling pathway [15, 16]. Therefore, the PI3K/Akt signaling pathway plays a critical role in tumor cell apoptosis and the discovery of lung cancer targeted drugs. However, the mechanism of how ASPP2 affects apoptosis is not yet clear.

Recently, a miRNA that could regulate the target protein by binding to the 3'-UTR has received extensive attention [17-19]. APPS2 regulation provided new opportunities for the treatment of cancer. Bioinformatics analysis revealed that miR-21 could target and regulate ASPP2, which has been predicted and validated in the research on glioma cells [20]. Additionally, miR-21 was overexpressed in osteosarcoma cells and positively correlated with the expression levels of miR-21 and its ability to invade cancer cells [21]. Thus, it is urgent to study the mechanism of ASPP2 mediated by miR-21 in lung cancer. Our study showed that the miR-21 inhibitor promoted apoptosis through inhibiting the PI3K/Akt/NF- κ B signaling pathway in NSCLC *in vitro* and *in vivo*. Moreover, downregulation of miR-21 suppressed cell migration and invasion, as well as the EMT signaling pathway in NSCLC cells. Additionally, the miR-21 inhibitor induced cell apoptosis via the caspase-dependent pathway in NSCLC cells. Our findings provided a novel molecular mechanism of miR-21 involving the PI3K/Akt/NF- κ B signaling pathway in NSCLC.

Materials and Methods

Medicines and reagents

The miR-21 mimic, the miR-21 inhibitor and the control inhibitor oligonucleotides were purchased from Ambion (Austin, TX). z-VAD-fmk was purchased from R&D Systems (Minneapolis, MN, USA). LY294002 was obtained from Cell Signaling Technology (Beverly, MA, USA). Lipofectamine 3000 was obtained from Invitrogen (Carlsbad, CA, USA). Apoptosis Detection Kit was purchased from BioLegend (San Diego, CA, USA). Bcl, Bax, p-Akt, Akt, P65, I κ B, ASPP2, E-cadherin, N-cadherin and Vimentin antibodies were obtained from Cell Signaling Technology (Beverly, MA, USA).

Cell culture

HBE and A549 cell lines were obtained from Cell Bank of CAS (Chinese Academy of Sciences) (Shanghai, China). RPMI-1640 medium supplemented with 100 U/mL penicillin, 100 μ g/mL streptomycin and 10% fetal bovine serum were used to maintain the cells in a humidified incubator at 37°C in an atmosphere of 5 % CO₂.

Apoptosis assay

Apoptosis assay was performed using the apoptosis detection kit according to the manufacturer's instructions. Cells were treated with the miR-21 inhibitor for 48 h, and stained with 7-aminoactinomycin D (7-AAD) and Annexin V-fluorescein isothiocyanate (FITC), and analyzed by flow cytometry on a FACSCalibur (BD Biosciences, CA, USA).

RNA extraction and quantitative real-time polymerase chain reaction analysis

Total RNA was isolated using TRIzol reagent (Invitrogen) as the manufacturer's instructions. For analysis of miR-21 expression, Real-Time PCR analyses were carried out using TaqMan miR assays (Applied

Biosystems) according to the manufacturer's instructions. The miR-21 primers were used as followed: Forward primer, 5'-GGACTAGCTTATCAGACTG-3'; Reverse primer, 5'-CATCAGATG CGTTGCGTA-3'. GAPDH: Forward primer, 5'-ATCTGGAGTTTACCGCTGG-3'; Reverse primer, 5'-TACCGATGTCTGGTAGACGAT-3'. PCR conditions were: 95°C for 10 sec, followed by 40 cycles each containing 95°C denature for 10 sec, 58°C annealing 30 sec and 72°C 10 sec. Standard curve was plotted depended on CT values of standards. The expression level was quantified using the ABI Prism 7900HT Sequence Detection System (Applied Biosystems).

Western blot

Protein was isolated from 10% sodium dodecyl sulfate-polyacrylamide gel (SDS-PAGE), then transferred on to a nitrocellulose membrane (Bio-Rad, Hercules, USA). The primary antibodies and secondary antibodies conjugated with horseradish peroxidase (Cell Signaling Technology, MA, USA) were used to detect the bands. The results were visualized by utilizing blotting analysis system (Thermo Fisher Scientific, MA, USA).

Migration and invasion assays

The migration and invasion ability were detected using a Transwell Boyden Chamber (8-mm pore size, BD Biosciences). For the cell invasion assay, the upper compartment of the chambers was coated with matrigel (BD Biosciences, Franklin Lakes, NJ, USA). The lower compartment of the chambers contained media with 10% FBS. Data was expressed as the mean number of cells that invaded through the Matrigel.

Caspase activity

The activation of caspase-3, 8, 9 were detected by caspase activity assay. After incubation of the miR-21 inhibitor for 24 h, 10 µL of lysis buffer was added. Cell lysate was incubated with 5 µL of chromogenic substrate at 37°C under dark conditions for 20 min. The results were detected using a plate reader under 560 nm.

Luciferase Reporter assays

The promoters of ASPP2 and NF-κB were cloned in pGL 3.0 luciferase reporter plasmids. The cells were cotransfected with pRL-CMV renilla luciferase reporter and the pGL 3.0 luciferase reporter plasmid containing the promoters of ASPP2 or NF-κB. The luciferases were measured by a dual luciferase reporter assay system (Promega).

Adenovirus vector construction, infection and transfection

HEK-293T cells were incubated in culture dish. After HEK-293T cells were seeded overnight, the shuttle vector containing Akt and Ikkβ and pAd vector were cotransfected into HEK-293T by Lipofectamine 3000 (Invitrogen, CA, USA). Viral lysates were harvested, purified and tittered. After cells were seeded in plate for 24 h. Adenovirus vectors were added into the medium and infected the cells.

Animals

6-week old BALB/c nude mice were provided by the Animal Center of the Second Military Medical University. All animals were kept in a standard laboratory diet and water ad libitum. The protocols of animal use and care conformed to Guide for the Care and Use of Laboratory Animals from the National Institutes of Health, and all experimental protocols described in this study were approved by the Animal Care Committee of the Zhejiang Provincial People's Hospital. Thirty nude mice bearing A549 tumor xenografts were divided into five groups randomly: Control, miR-21 inhibitor, miR-21 inhibitor + ASPP2 shRNA, miR-21 inhibitor + Akt, and miR-21 inhibitor + LY. The volumes of the tumors were measured every week.

TUNEL staining

Tissues were embedded, sectioned, and deparaffinized. The specimens were incubated with proteinase K (40 µg/mL) for 1 hour at 37 °C, then treated with 2% H₂O₂ in distilled water for 30 min at 37 °C. After enzymatic reaction, sections were washed with PBS, incubated with anti-digoxigenin peroxidase conjugate for 30 min at 37 °C in a humidified chamber. The sections were stained with diaminobenzidine and counterstained with hematoxylin, and observed under a light microscope.

Statistical analysis

The results were expressed as the mean $\pm$ SD and performed at least three times. Differences between two groups were analyzed by Student's t-test or one-way analysis of variance with Dunnett's test. $P < 0.05$ were considered statistically significant.

Results

The miR-21 inhibitor induced cell apoptosis in A549 cells

We first evaluated the miR-21 levels in lung cancer A549 cells. Q-PCR results suggested that miR-21 was overexpressed in A549 cells compared with normal human bronchial epithelial (HBE) cells (Fig. 1a). Additionally, the expression of miR-21 significantly decreased in the presence of the miR-21 inhibitor (Fig. 1a). Subsequently, flow cytometry was employed to detect the effects of miR-21 on the apoptosis of A549 cells. The results showed that increased apoptosis in A549 cells was observed, indicating that the miR-21 inhibitor could induce apoptosis in A549 cells (Fig. 1b). Western blotting further confirmed this phenomenon, as Bcl-2 expression levels were reduced and the Bax levels obviously increased after A549 cells were treated with a miR-21 inhibitor (Fig. 1c). The transwell migration and invasion results revealed that the inhibitor of miR-21 could go against metastasis and the invasion of A549 cells (Fig. 1d and e). We then investigated the effect of the miR-21 inhibitor on the EMT signaling of A549 cells. The EMT-associated proteins were monitored and the result showed that the expression

Fig. 1. miR-21 inhibitor induced cell apoptosis in lung cancer cells. (a) The miR-21 expression was detected in normal and lung cancer cells. (b) miR-21 inhibitor induced cell apoptosis in A549 cells. $^{**}P < 0.01$ VS HBE, $^{#}P < 0.01$ VS Blank. (c) The protein expressions of Bcl-2, Bax were detected following miR-21 inhibitor treatment in lung cancer cells. (d) Transwell migration assays analysis of lung cancer cells treatment with miR-21 inhibitor. $^{**}P < 0.01$ VS Blank. (e) Transwell invasion assays analysis of lung cancer cells treatment with miR-21 inhibitor. $^{**}P < 0.01$ VS Blank. (f) Western blotting analysis for protein expressions of E-cadherin, N-cadherin and Vimentin were measured in A549 cells following treatment with miR-21 inhibitor. (g) Caspase-3, 8, 9 activities were detected after treatment with miR-21 inhibitor in A549 cells. $^{**}P < 0.01$ VS Blank, $^{***}P < 0.001$ VS Blank. (h) Cell apoptosis was detected for A549 cells treated with miR-21 inhibitor followed by the pretreatment with z-VAD-fmk for 1 h. $^{***}P < 0.001$ VS miR-21 inhibitor treatment.

level of E-cadherin was decreased, while the N-cadherin and Vimentin expression levels were increased in the miR-21 inhibitor treated cells (Fig. 1f). We also tested the activity of Caspase-3, 8, 9 after treatment with the miR-21 inhibitor in A549 cells. The activity of Caspase-3, 8, 9 were dramatically increased in the presence of the miR-21 inhibitor (Fig. 1g). Moreover, an obvious increase of apoptosis was observed in A549 cells after incubation with the miR-21 inhibitor using analysis of flow cytometry (Fig. 1h). However, the apoptosis was significantly reduced in A549 cells when preincubated with the caspase inhibitor, Z-VAD-FMK.

miR-21 decreased the expression of ASPP2 in A549 cells

We next examined whether miR-21 regulate the ASPP2 expression in A549 cells. A549 cells were incubated with the miR-21 mimic or the miR-21 inhibitor for 24 h, the cell lysates were collected and analyzed by Western blotting assay. The result showed that the ASPP2 expression level was increased in 549 cells when treated with the miR-21 inhibitor, whereas reduced drastically in the presence of the miR-21 mimic (Fig. 2a). To investigate the target of miR-21, the luciferase reporter vector containing the entire wild-type 3'UTR of ASPP2 or the mutant 3'UTR with a 5-nucleotide mutation was transfected into A549 cells. As expected, miR-21 reduced the ASPP2-driven gene expression, and the miR-21 inhibitor significantly enhanced the luciferase activity (Fig. 2b). However, the activity result showed no obvious change in the mut transfected A549 cells (Fig. 2b). To further confirmed the relation between miR-21 and ASPP2 in A549 cells, the ASPP2 knockdown assay was carried out (Fig. 2c). The results showed that apoptosis was markedly inhibited in the ASPP2 knockdown cells (Fig. 2d).

Effect of miR-21 on the PI3K/AKT and NF- κ B signaling pathways in A549 cells

To explore the influence of the miR-21 inhibitor on the PI3K/AKT pathway, the LY294002 (LY), a reported highly selective inhibitor of phosphatidylinositol 3 (PI3) kinase, was used to inhibit the PI3K/AKT pathway. The miR-21 inhibitor decreased the p-Akt expression level, even in the case of overexpression of Akt (Fig. 3a). Subsequently, we also investigated the effect of the miR-21 inhibitor on apoptosis in A549 cells. The result suggested that miR-21 inhibitor could induce apoptosis through inhibiting the expression of Akt (Fig. 3b). The expression level of Bcl2 was reduced, while the Bax expression level was increased when incubation with the miR-21 inhibitor (Fig. 3c). Based on these

Fig. 2. miR-21 inhibited ASPP2 expression in lung cancer cells. (a) The protein expression of ASPP2 following treatment with miR-21 or miR-21 inhibitor in A549 cells. (b) Luciferase reporter assay revealed the effect of miR-134 or miR-21 inhibitor on ASPP2 3'UTR luciferase activity in A549 cells. **P<0.01, ***P<0.001 (c) The protein expression of ASPP2 was detected following ASPP2 shRNA treatment in A549 cells. (d) ASPP2 shRNA treatment reversed miR-21 inhibitor induced cell apoptosis in A549 cells. ***P<0.001 VS miR-21 inhibitor treatment.

findings, the expression level of NF- κ B was also detected in the miR-21 inhibitor treated A549 cells. As expected, the p65 expression level was decreased by the miR-21 inhibitor treatment in a time dependent manner (Fig. 3d). This result was further confirmed by detecting the level of downstream protein of NF- κ B. The IKK β expression level was obviously reduced in the presence of the miR-21 inhibitor in A549 cells (Fig. 3e). Furthermore, up-regulation of IKK β significantly inhibited the apoptosis induced by miR-21 (Fig. 3f). In addition, IKK β could release the regulation of the Bcl2 and BAX mediated by the miR-21 inhibitor (Fig. 3g).

The relationship between PI3K/AKT and NF- κ B signaling in A549 cells

To further investigate the relationship between PI3K/AKT and NF- κ B signaling after the miR-21 inhibitor treatment, we constructed a luciferase reporter vector containing the entire wild-type 3'UTR of NF- κ B. The wild-type plasmid was cotransfected into A549 cells along with the Akt vectors and/or the miR-21 inhibitor. The results revealed that the miR-21 inhibitor reduced the NF- κ B-driven gene expression, and the Akt vector transfection significantly enhanced the luciferase activity induced by the miR-21 inhibitor (Fig. 4b).

In addition, the protein expression level of P65 was obviously enhanced following the Akt vector transfection, while the LY and the miR-21 inhibitor treatment reduced the P65 protein expression compared with the miR-21 inhibitor alone treatment in A549 cells (Fig. 4a).

The miR-21 inhibitor induced lung cancer cell apoptosis in vivo

To further evaluate the tumor suppressor role of the miR-21 inhibitor, we determined whether shikonin could inhibit the development of tumor growth in a tumor xenograft model. Thirty nude mice bearing A549 tumor xenografts were divided into five groups

Fig. 3. miR-21 inhibitor regulates PI3K/AKT and NF- κ B signaling in lung cancer cells. (a) The protein expression levels of p-Akt and total Akt were detected in A549 cells. (b) Cell apoptosis was detected for A549 cells treated with or without miR-21 inhibitor followed by the pretreatment with LY or transfection with Akt adenovirus vectors. ** $P < 0.01$ VS Control, *** $P < 0.001$ VS Control. # $P < 0.01$ VS miR-21 inhibitor alone treatment. (c) The protein expressions of Bcl-2, Bax were detected following miR-21 inhibitor treatment after transfection with Akt adenovirus vectors or LY. (d) The protein expression of P65 was detected following miR-21 inhibitor in a time dependent manner. (e) The protein expression of Ikk β was detected following miR-21 inhibitor treatment and/or transfection with Ikk β adenovirus vectors. (f) Cell apoptosis for A549 cells treated with miR-21 inhibitor and/or transfection with Ikk β adenovirus vectors. ** $P < 0.01$ VS miR-21 inhibitor alone treatment. (g) Western blotting analysis for protein expressions of Bcl-2, Bax were measured in A549 cells following treatment with miR-21 inhibitor and/or transfection with Ikk β adenovirus vectors.

randomly: Control, miR-21 inhibitor, miR-21 inhibitor + ASPP2 shRNA, miR-21 inhibitor + Akt, and miR-21 inhibitor + LY. The volume of the tumors was measured every week. Tumor growth was significantly inhibited by the miR-21 inhibitor, and xenograft A549 cell growth in nude mice was most effectively inhibited by combined treatment with the miR-21 inhibitor and LY *in vivo* in comparison to the control group (Fig. 5a). To determine whether the tumor suppressor role of miR-21 inhibitor in A549 xenografts could be attributable to apoptosis induction, we performed a TUNEL assay. Consistent with the tumor volume outcome, the number of TUNEL-positive cells in tumor tissue was most dramatically increased in tumors from mice that received combined treatment with the miR-21 inhibitor and LY in comparison to the miR-21 inhibitor alone treatment group. The number of TUNEL-positive cells in tumor tissue was rescued in tumors from mice that received combined treatment with the miR-21 inhibitor and ASPP2 shRNA or Akt when compared with the miR-21 inhibitor alone treatment group (Fig. 5b and c). Additionally, activation of Bax, as well as inactivation of Bcl-2, p-AKT and P65 was observed following treatment with the miR-21 inhibitor or combined treatment with the miR-21 inhibitor and LY *in vivo* (Fig. 5d).

Fig. 4. The relationship between PI3K/AKT and NF-κB signaling in lung cancer cells. (a) The protein expression of P65 following treatment with or without miR-21 inhibitor after treatment with LY or transfection with Akt adenovirus vectors in A549 cells. (b) Luciferase reporter assay revealed the activation of NF-κB was induced by the miR-21 inhibitor treatment followed transfection with Akt adenovirus vectors in A549 cells. **P<0.01 VS miR-21 inhibitor alone treatment.

Fig. 5. miR-21 inhibitor induced apoptosis in vivo. (a) Tumor volume was measured followed with different treatments for indicated time points. (b, c) Apoptosis was detected using TUNEL staining for the lung tissue treated with different treatments. **P<0.01 VS miR-21 inhibitor alone treatment, ***P<0.001 VS miR-21 inhibitor alone treatment. (d) The protein expressions of Bcl-2, Bax, p-Akt, P65 were detected following with different treatments.

Discussion

miRNAs play a critical role in a multistep biological process including cell growth, differentiation and apoptosis [22-24]. Increasing evidence demonstrates that miRNAs were associated with the initiation and development process of non-small cell lung cancer (NSCLC) [25-27]. Recent studies demonstrated that miR-21 was up-regulated in non-small cell lung cancer [28]. In the present study, we showed that miR-21 was up-regulated in NSCLC cells compared with normal HBE cells. A significantly higher level of apoptosis was shown compared with the blank in NSCLC cells after down-regulation of miR-21 with miR-21 inhibitor. Moreover, the miR-21 inhibitor induces cell apoptosis through the caspase-dependent pathway. Previous studies showed that solasodine inhibited the invasion of human lung cancer cells through downregulation of miR-21 [29]. Our results revealed that the miR-21 inhibitor significantly inhibited migration and invasion, as well as the EMT signaling in NSCLC cells.

To investigate the function of miR-21 in NSCLC cells, we detected the activity of the ASPP2 promoter using the luciferase reporter assay by treating with miR-21 or miR-21 inhibitor in wild-type and mutant models. We found that luciferase and protein levels of ASPP2 were directly inhibited by miR-21. Meanwhile, down-regulation of ASPP2 could significantly abolish the apoptosis induced by the miR-21 inhibitor. To further explore the detailed mechanism of miR-21 in NSCLC cells, the PI3K/AKT and NF- κ B signaling pathways were tested using miR-21 inhibitor. A number of studies showed that PI3K/AKT was involved in human lung cancer [30-33]. Previous studies identified activation of the PI3K/AKT pathway and observed it in human lung carcinomas [34, 35]. Another study showed that PI3K/Akt regulated the expression of angiogenic factors in A549 lung cancer cells [36]. Our results showed that the miR-21 inhibitor could suppress the phosphorylation of Akt. Interestingly, the Akt inhibitor LY could enhance the effect of the miR-21 inhibitor on apoptosis. However, Akt adenovirus vector infection significantly suppressed the effect of the miR-21 inhibitor on apoptosis in NSCLC cells. Consistently, LY obviously down-regulated the apoptotic proteins induced by the miR-21 inhibitor. A previous study demonstrated that XPO1 inhibitor sensitivity to lung cancer was involved in the inhibition of NF- κ B transcription factor activity [37]. Kashyap et al. indicated that Selinexor showed significant anticancer activity against osteosarcoma by NF- κ B inhibition [38]. We found that the miR-21 inhibitor inhibited the expression of P65 in a time-dependent manner. Furthermore, up-regulation of NF- κ B could decrease the apoptosis and relative protein expression levels. Interestingly, LY treatment obviously inhibited NF- κ B protein expression. Moreover, adenovirus vector infection could restore the activity of NF- κ B inhibited by miR-21 inhibitor using luciferase detection in NSCLC cells, which suggested that the miR-21 inhibitor induces cell apoptosis through PI3K/AKT/NF- κ B in NSCLC cells.

In conclusion, our study demonstrated that the miR-21 inhibitor promoted apoptosis through inhibiting the PI3K/Akt/NF- κ B signaling pathway in NSCLC *in vitro* and *in vivo*. Meanwhile, ASPP2 was a direct target of miR-21 in NSCLC cells. Down-regulation of miR-21 suppressed cell migration and invasion, as well as the EMT signaling pathway in NSCLC cells. Moreover, the miR-21 inhibitor induces cell apoptosis by the caspase-dependent pathway in NSCLC cells. These findings provided a novel molecular mechanism, which could be a new potential therapeutic target in NSCLC.

Disclosure Statement

No conflict of interests exists.

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