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Original Article

Antagonism of miR-21 reverses radiation-induced EMT in alveolar epithelial cells via PI3K/Akt pathway

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Abstract: Radiation-induced lung injury (RILI) is a common complication after thoracic radiotherapy and epithelial-to-mesenchymal transition (EMT) an important change among this process. The aim of this study was to determine the role of miR-21 in radiation-induced EMT in alveolar epithelial type II cells RLE-6TN and explored the underlying molecular mechanism. The results showed that treating RLE-6TN cells with 8 Gy of X-ray promoted EMT and resulted in up-regulation of miR-21. Transfection of Rle-6TN cells with miR-21 inhibitor before IR caused an increase in expression of epithelial marker e-cadherin, as well as a decrease in the mesenchymal markers α-smooth muscle actin and vimentin. It indicated that downregulation of miR-21 in RLE-6TN cells inhibited the radiation-induced EMT. Moreover, miR-21 inhibitor decreased the progress of this EMT accompanied by a decrease of phosphorylated-Akt protein level. However, PI3K activator IGF-1 reversed the suppression of phosphorylated-Akt and promoted the radiation-induced EMT by miR-21 knockdown. In addition, we found a dose-dependent relationship between PI3K inhibitor LY294002 and radiation-induced EMT. In total, we concluded that antagonism of mir-21 reversed radiation-induced EMT in alveolar epithelial cells via PI3K/Akt pathway. This might be a much promising target in the cure the radiation-induced lung injury.

Keywords: RILI, EMT, miR-21, PI3K/Akt

Introduction

Radiation-induced lung injury (RILI) is the common complication of thoracic radiotherapy. Because of the lung is one of the most sensitive tissues to ionizing radiation, the damage to normal lung tissue remains a major obstacle in the treatment of lung cancer. It contains radiation pneumonia in the early period and radiation pulmonary fibrosis in the later period [1, 2]. Generally, we considered that pulmonary fibrosis is characterized by alveolar epithelial cell injury, leading to the accumulation of fibroblasts, myofibroblasts, collagen and other extracellular matrix proteins and resulting in impaired lung function [3, 4]. But recent evidence suggests that injured epithelial cells may directly turn to myofibroblasts by epithelial-mesenchymal transition (EMT) [5]. This transition is characterized by the loss of epithelial markers such as E-cadherin and the increase of mesenchymal markers including α-SMA, vimentin. Signaling pathway such as ERK/Snail, PI3k/Akt involved in the process [6]. Studies have confirmed that several microRNAs regulate the process of EMT, such as miR-200 family, let-7d, miR-21 [7].

MicroRNAs (miRNAs) are highly conserved, small, non-coding RNAs of approximately 19-25 nucleotides in length, which function as versatile regulation of gene expression [8]. These small non-coding RNAs negatively regulate the expression of thousands of genes through either inhibiting translation or initiating specific degradation of target mRNAs by binding to complementary sequences in the 3' untranslated regions (3'UTRs). MiRNAs are vital mediators in various cellular processes such as development, proliferation, differentiation, migration, apoptosis and cell death [9, 10]. Expression change of miRNAs has been shown to be associated with a wide variety of diseases, including lung fibrosis [11, 12]. Furthermore, it is found

that the expression of miRNAs has changed either in peripheral blood cells of radiotherapy patients and in the cancer cells treated with ionizing radiation [13, 14]. And in an animal experiment, the miRNAs in the lung of the rat with ionizing radiation has changed, including the up-regulation of miR-21 [15].

MiR-21 is one of the miRNAs who located on chromosome 17q23.2, which controls a wide range of biological processes, including cell growth, proliferation, migration, invasion, and survival [16]. It has been reported to be overexpressed in almost all types of human cancers [17]. Besides, miR-21 related to many other diseases especially fibrosis [18]. It is reported that miR-21 mediated fibrogenic activation of pulmonary fibroblasts and lung fibrosis [19]. Many studies have shown the relation between miR-21 and radiation, such as circulating mir-21 upregulated in the patients who treated with radiotherapy, miR-21 may promote radioresistance of cancer and inhibition of miR-21 may represent effective approaches for reversing this radioresistance [20]. Therefore, in this study, we investigate the role of miR-21 in the radiation-induced EMT in alveolar epithelial cells for the sake of the therapy of radiation-induced lung injury.

Materials and methods

Cell culture

RIE-6TN cells, a rat alveolar type II epithelial cell line, were obtained from the Chinese Academy of Sciences and cultured in RPMI-1640 medium (GE Healthcare Life Scinences/HyClone™ Laboratories, Logan, UT) containing 10% fetal bovine serum (FBS) (GE Healthcare Life Scinences/HyClone™ Laboratories, Logan, UT), 100 IU/ml penicillin and 100 μg/ml streptomycin at 37°C with 5% CO_2 in air. Growth medium was changed every 48 hours. Cells were subcultured every 3-5 days. Suspensions of RLE-6TN cells were obtained from mostly confluent cultures (about 80-90%) using Trypsin/EDTA solution.

Transfection

Rno-miR-21 inhibitors, miRNA inhibitor negative controls, rno-miR-21 mimics and miRNA mimic negative controls were designed and synthesized by Guangzhou RiboBio Co., Ltd. (Guangzhou, China). The transfection of miRNAs was performed using Lipofectamine® 2000 (Invitrogen™, Carlsbad, CA). In six-well plates (Costar™), cells were plated per well at 40-60% confluence 24 h prior to the transfection. MiR-21 inhibitors, mimics, the negative controls and cy3-labeled scrambled were transfected into the cells at a final concentration of 100 nM with 5 μl of Lipofectamine 2000 according to the manufacturer's protocol. The medium was replaced with new culture medium at 4-6 h after transfection. The initial calibration of transfection efficiency was done by demonstrating that, after transfection with cy3-labeledscrambled microRNA, over 80% of the cells expressed cy3.

Irradiation conditions

Linear accelerator producing 6MV X-ray beams were provided by Zhongnan Hospital of Wuhan University (Wuhan, China). The source-to-skin distance (SSD) was 100 cm and the dose rate was 200 cGy/min. Packing material 3 cm thick was placed around and underneath the culture dishes. Then the cells that after 24 h post transfection were treated with a single dose of 8 Gy. All irradiations were performed at room temperature.

RNA isolated and quantitative real-time PCR

Total RNA including the miRNA fraction was isolated from cell lines using TRIzol® reagent (Invitrogen) according to the manufacturer's protocol. cDNA was synthesized from total RNA following the manufacturer's protocols from Applied Biosystems® (Carsbad, CA). And miRNA reverse transcription into cDNA was performed following the manufacturer's protocol form Guangzhou RiboBio (Guangzhou, China). Real-time PCR was performed with SYBR® Premix Ex Taq™ (Takara Bio Inc., Shiga, Japan) in a 25 μl reaction volume (12.5 μl SYBR Premix Ex Taq, 0.5 μl ROX™ Reference Dye II, 1 μl forward primer, 1 μl reverse primer and 2 μl cDNA template) on a MJ Opticon Monitor Chromo4™ instrument (Bio-Rad Laboratories Inc., Hercules, CA). The following protocol was used for miRNA: 95°C for 20 s; 40 cycles of 95°C for 10 s, 60°C for 20 s and 70°C for 10 s. And the following protocol was used for other RNAs: 93°C for 4 min; 40 cycles of 93°C for 30 s, 60°C for 30 s and 70°C for 30 s. The primers for miR-21 and u6 for real-time PCR were designed and

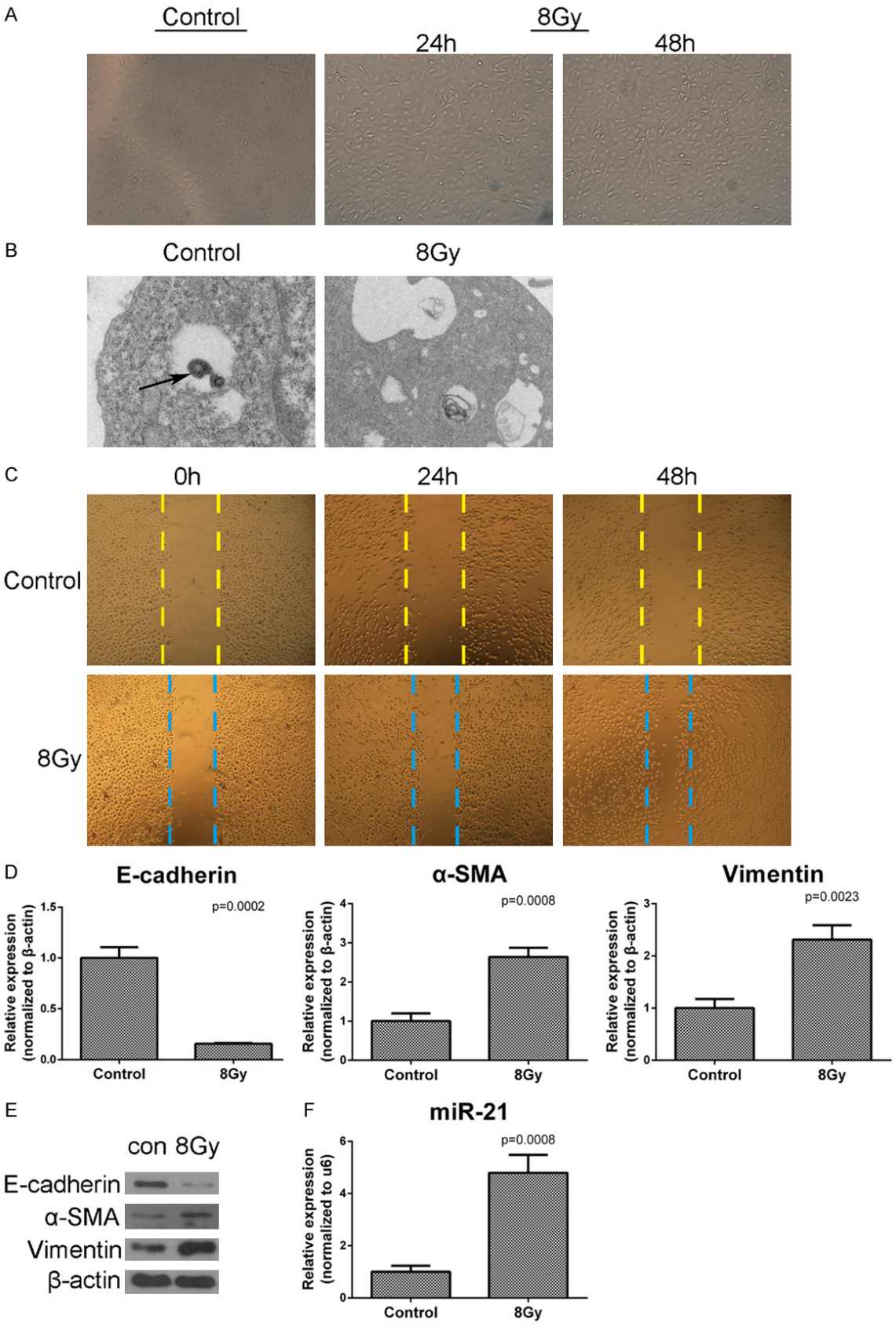

Figure 1. The effects of irradiation in RLE-6TN cells. RLE-6TN cells were irradiated with 8 Gy of X-ray, cell morphology, cell subcellular structure and cells immigration were observed, protein and mRNA level were examined. A. The

cell morphology was observed by light microscope (×100) at 24 h and 48 h postirradiation. B. The cell subcellular structure was observed by electron microscope (×15.0K). The osmiophilic lamellar body of normal cell was clearly visible (arrow) but it disappeared after irradiation. C. The cells immigration was detected by Scratch Assay and observed by light microscope (×40). The equidistance lines in the same color were drawn for easily observing. D. After 24 h postirradiation, cells were collected and mRNA expression of E-cadherin, α-SMA and vimentin were detected by real-time PCR. Results are representative of three independent experiments. β-actin served as the loading control. Data are mean ± SD, n = 3, $P < 0.05$. E. The protein expression of E-cadherin, α-SMA and Vimentin were detected by western blot after 48 h postirradiation. β-actin served as the loading control. F. The miR-21 expression as detected by real-time PCR ($P < 0.05$).

synthesized by Guangzhou RiboBio (Guangzhou, China). The other primers were designed and synthesized by GenePharma (Shanghai, China): E-cadherin (forward primer 5'-TGACTACTACTTGAACGAATGGG-3', reverse primer 5'-GGAAGGGAGCTGAAAAACCAC-3'); α-SMA (forward primer 5'-TGACGCTGAAGTATCCGATAGA-3', reverse primer 5'-GTACGTCCAGAGAGGCATAGAGG-3'); Vimentin (forward primer 5'-ATGTGGATGTTTCCAAGCCTGAC-3', reverse primer 5'-GAGTGGGTATCAACCAGAGGGAGT-3'); β-actin (forward primer 5'-AGAAAATCTGGCACCACACC-3', reverse primer 5'-CCATCTCTTGCTCGAAGCTCC-3').

Western blots analysis

Cells were prepared at 48 h postirradiation, washed with PBS twice and lysed using RIPA buffer containing 50 mM Tris (pH 7.4), 150 mM NaCl, 1% NP-40, 0.25% sodium deoxycholate, 1% protease inhibitor cocktail (Beyotime, China), 1% Na_3VO_4 and 1% phosphatase inhibitor cocktail (Roche Diagnostics Corp., Indianapolis, IN). The proteins were separated by SDS-PAGE and then transferred using a PVDF membrane (Millipore, Billerica, MS) by Western blotting. The nonspecific sites were blocked in 5% nonfat dry milk for 1-2 h at room temperature. In this study, antibodies directed against E-cadherin, α-SMA, Vimentin, PTEN, Akt, p-Akt (each at 1:500; Proteintech Group Inc., Chicago, IL) and β-actin (1:2000; Cell Signaling Technology®, Danvers, MA) were used. The secondary antibodies were goat anti-rabbit horseradish peroxidase or goat anti-mouse horseradish peroxidase (Bio-Rad Laboratories Inc.) used at 1:20,000 or 1:10,000, respectively.

Scratch assay

RIE-6TN cells were cultured in six-well dishes and treated or not as described above. The scratch wounds were creased using a p-10 micropipette tip into confluent cells. Two vertical lines and one horizontal line were scored per well to simulate a "wound" by scratching the culture. After scratching, cells were washed twice with PBS to remove cell debris and supplemented with regular growth medium. Images were captured by phase-contrast microscopy at 0, 24, and 48 h after wounding.

Statistical analysis

The results were performed as the mean ± SD from at least three independent experiments. The data obtained in experiments were analyzed by one-way ANOVA and unpaired two-tailed t tests. *P* values < 0.05 were considered statistically significant.

Results

Radiation induced EMT and up-regulated-miR-21

RLE-6TN cells were irradiated with a single dose of 8 Gy X-ray which is an appropriate dose to induce EMT [21]. Cell morphology was observed at 24 h and 48 h postirradiation under the optical microscopy. Cells irradiated with 8Gy lost their cuboidal appearance and showed an elongated mesenchymal-like morphology (**Figure 1A**). Then we observed the cells under the electron microscope, the osmiophilic lamellar body disappeared in the cells after irradiation (**Figure 1B**). In scratch assay, we found that the cells irradiated were quicker to close a gap than the cells untreated (**Figure 1C**). Furthermore, we harvested the cells at 24 h postirradiation and examined the mRNA expression of EMT associated proteins by real-time PCR. We found significant decrease of E-cadherin, increase of α-SMA and vimentin in mRNA levels (**Figure 1D**). Then we examined the protein level expression of these proteins by western blot at 48 h postirradiation (**Figure 1E**). Examined the expression of miR-21, we found significant increase of miR-21 after irradiation (**Figure 1F**).

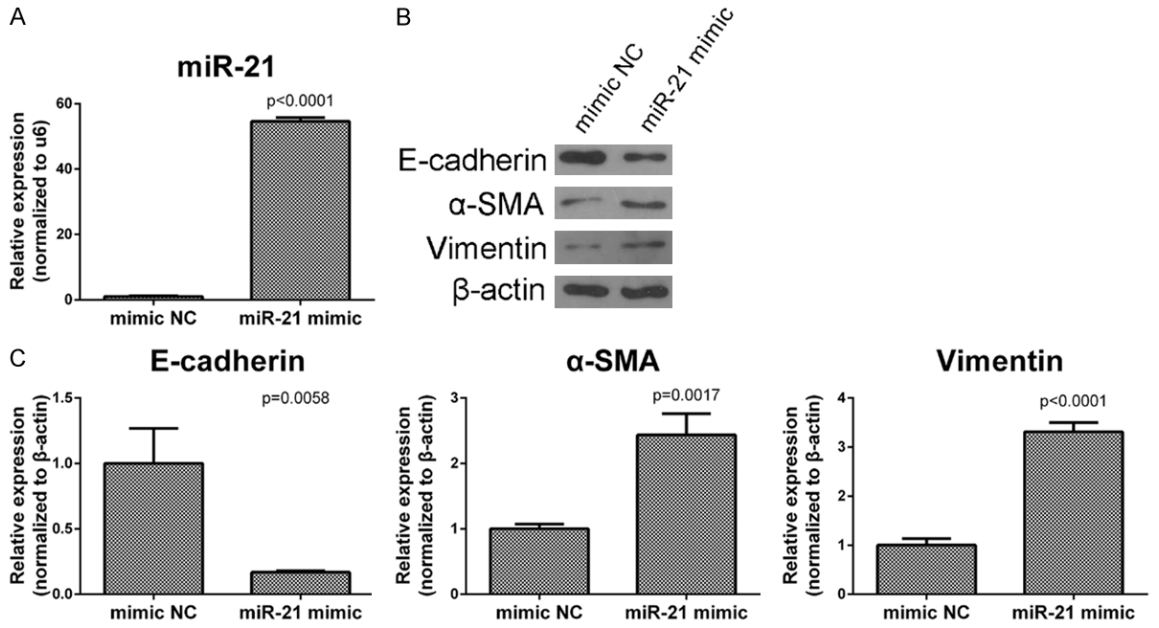

Figure 2. Overexpression of miR-21 induced EMT in RLE-6TN cells. Cells were transfected with miR-21 mimic or mimic NC. A. The transfection efficiency of miR-21 mimic in normal RLE-6TN cells was detected by real-time PCR after 24 h posttransfection. B. After 48 h posttransfection, the protein expression of E-cadherin, α-SMA and Vimentin were examined by western blot. C. After 24 h posttransfection, cells were collected and mRNA expression of E-cadherin, α-SMA and Vimentin were detected by real-time PCR. Data are mean ± SD, n = 3, P < 0.05.

Overexpression of miR-21 increased EMT

Transfected miR-21 mimic or mimic NC in RLE-6TN cells and examined the expression of miR-21, we confirmed the transfection efficiency (**Figure 2A**). Then, we harvested the cells at 48 h after transfection and examined the EMT associated proteins by Western blots. Then we examined the gene expression of these proteins at 24 h after transfection by real-time PCR. The results show that with transfection of miR-21 mimic the expression of α-SMA and vimentin get a significant increase and the expression of E-cadherin also had a significant decrease in both protein and mRNA levels (**Figure 3C**).

Inhibition of miR-21 reduced radiation-induced EMT

Transfection of miR-21 inhibitor in RLE-6TN cells resulted in a significant decrease of its expression compared with endogenous levels of this miRNA (**Figure 3A**). The cells transfected with either miR-21 inhibitor or inhibitor NC were irradiated with 8 Gy at 24 h after transfection. Cell morphology was observed at 48 h postirradiation under the optical microscope. While the cells transfected with miR-21 inhibitor NC lost their cuboidal appearance and showed an elongated mesenchymal-like morphology as same as the only irradiation group, the cells transfected with miR-21 inhibitor reversed the EMT like change (**Figure 3B**). Then, we harvested the cells at 24 h postirradiation and examined the gene expression of EMT associated proteins by real-time PCR. Then we examined the protein level expression of these proteins at 48 h postirradiation by Western blots. With transfection of miR-21 inhibitor, the expression of α-SMA and vimentin get a significant decrease and the expression of E-cadherin get a significant increase compared with the cells transfected with inhibitor NC in both protein and mRNA levels (**Figure 3C-F**). In scratch assay, the cells transfected with miR-21 inhibitor were slower to close a gap than the inhibitor NC group at 24 h and 48 h postirradiation (**Figure 3G**).

Inhibition of miR-21 attenuated PI3K/Akt pathway activation after irradiation

The activation of PI3K/Akt signaling pathway was measured by Akt phosphorylation on Ser473. By western blot, we found that the

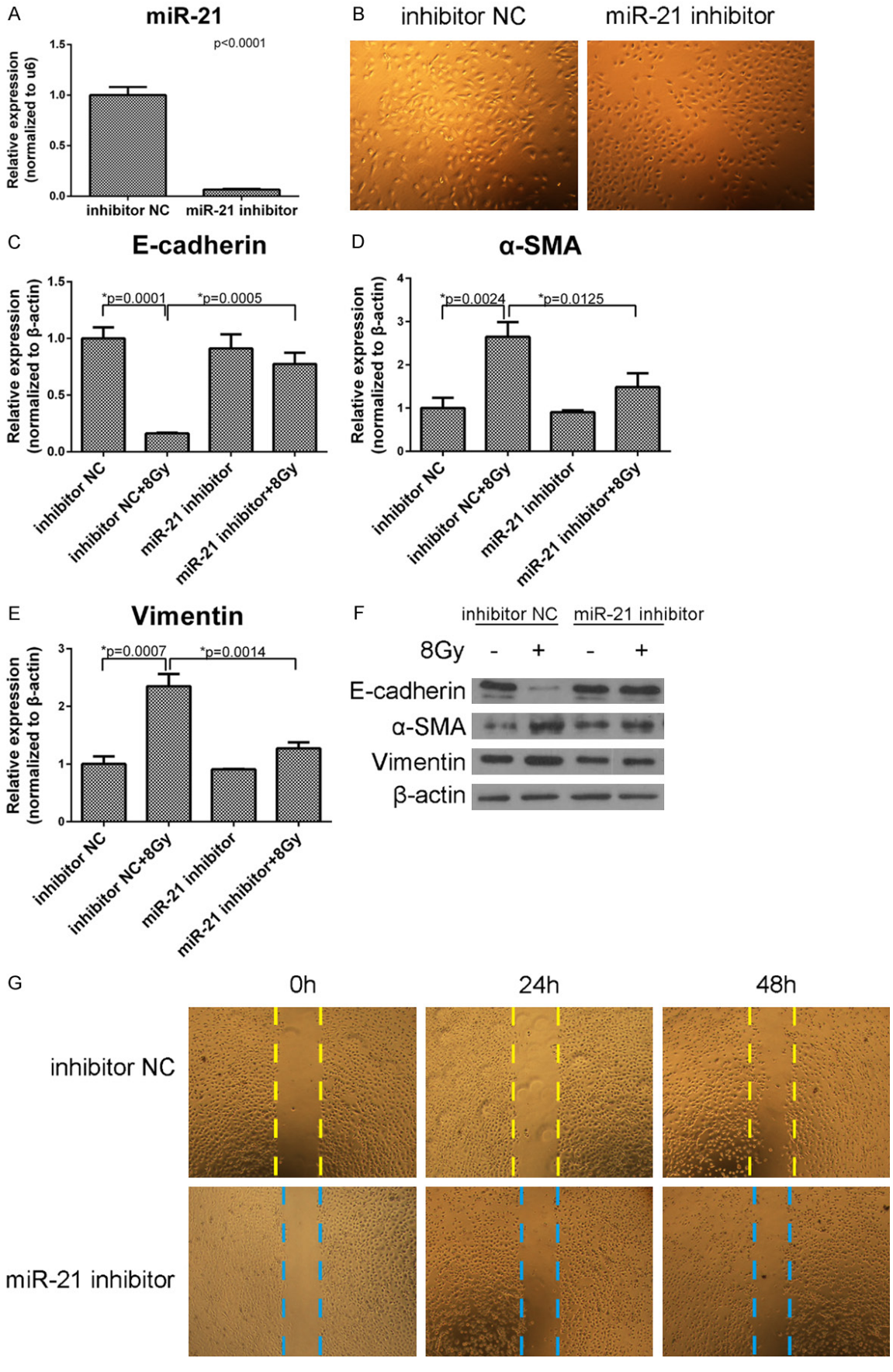
A
miR-21
p<0.0001
Relative expression (normalized to u6)
1.5
1.0
0.5
0.0
inhibitor NC
miR-21 inhibitor
B
inhibitor NC
miR-21 inhibitor
C
E-cadherin
*p=0.0001
*p=0.0005
Relative expression (normalized to β-actin)
inhibitor NC
inhibitor NC+8Gy
miR-21 inhibitor
miR-21 inhibitor+8Gy
D
α-SMA
*p=0.0024
*p=0.0125
Relative expression (normalized to β-actin)
inhibitor NC
inhibitor NC+8Gy
miR-21 inhibitor
miR-21 inhibitor+8Gy
E
Vimentin
*p=0.0007
*p=0.0014
Relative expression (normalized to β-actin)
inhibitor NC
inhibitor NC+8Gy
miR-21 inhibitor
miR-21 inhibitor+8Gy
F
inhibitor NC
miR-21 inhibitor
8Gy - + - +
E-cadherin
α-SMA
Vimentin
β-actin
G
0h
24h
48h
inhibitor NC
miR-21 inhibitor

Figure 3. The effects of miR-21 inhibitor in radiation-induced EMT in RLE-6TN cells. Cells were irradiated with 8Gy after miR-21 inhibitor or inhibitor NC was transfected. A. The transfection efficiency of miR-21 inhibitor in normal RLE-6TN cells was detected by real-time PCR after 24 h posttransfection. B. The changes in cell morphology were observed by light microscope (×100). C-E. After 24 h postirradiation, cells were collected and mRNA expression of E-cadherin, α-SMA and Vimentin were detected by real-time PCR. Data are mean ± SD, n = 3, P < 0.05. F. After 48 h postirradiation, the protein expression of E-cadherin, α-SMA and Vimentin were examined by western blot. G. The cells immigration of RLE-6TN cells were detected by Scratch Assay and observed by light microscope (×40).

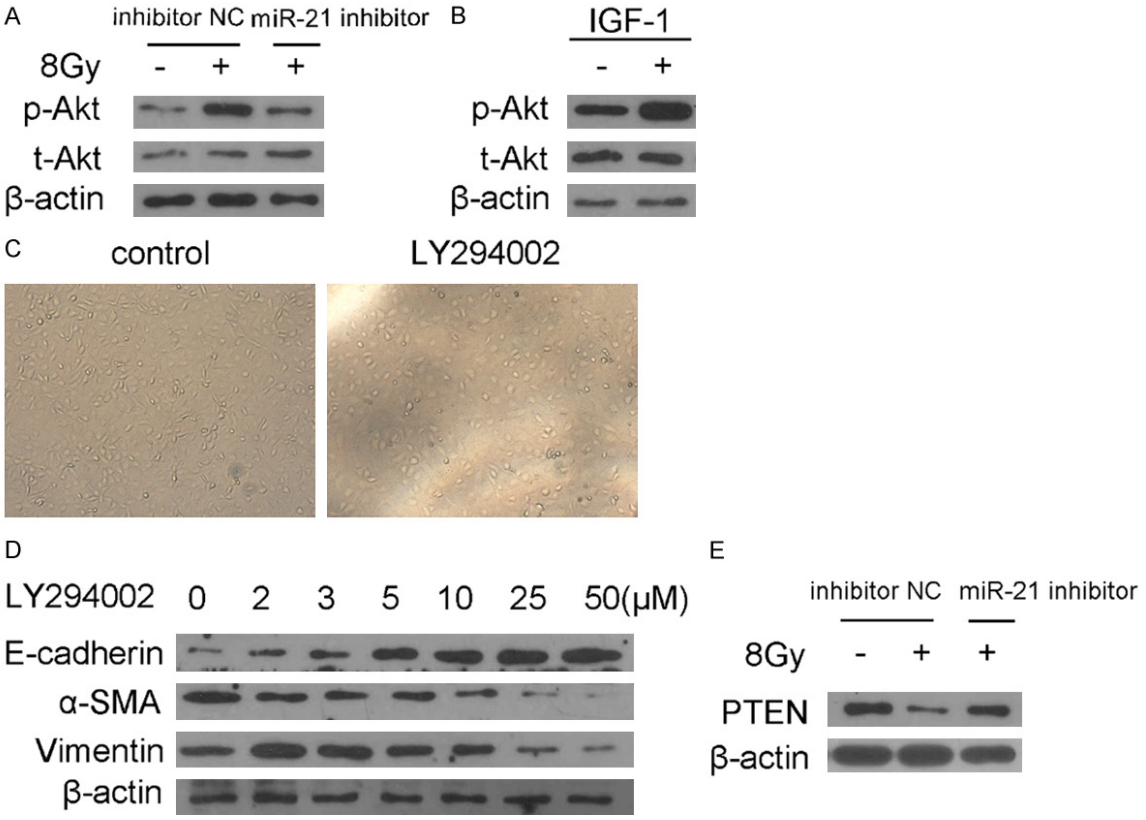

Figure 4. PI3K/Akt pathway mediated radiation-induced EMT. A. Cells were irradiated with 8 Gy after miR-21 inhibitor or inhibitor NC was transfected. The protein expression of p-Akt and t-Akt was examined by western blot. B. With or without 10 ng/ml PI3K activator IGF-1, the protein expression of p-Akt and t-Akt was measured by western blot. C. Cells were treated with PI3K inhibitor LY294002 50 μM or not without miR-21 before irradiation. The changes in cell morphology were observed by light microscope (×100). D. Cells were treated with PI3K inhibitor LY294002 by concentration gradient before irradiation. The protein expression of E-cadherin, α-SMA and Vimentin was examined by western blot. E. Cells were treated with 8 Gy after miR-21 inhibitor or inhibitor NC was transfected. The protein expression of PTEN was examined by western blot.

endogenous level of phospho-Akt expression (Ser473) in miR-21 inhibitor transfected RLE-6TN cells was downregulated compared with that in inhibitor NC transfected RLE-6TN cells after irradiation (**Figure 4A**). Interestingly, phospho-Akt (Ser473) expression was significantly increased in the case of being treated with IGF-1 [22], a PI3K activator, in miR-21 inhibitor transfected RLE-6TN cells after irradiation (**Figure 4B**). This suggested that activation of PI3K/Akt signaling pathway by irradiation in RLE-6TN cells was suppressed by knockdown of miR-21, and the suppression was reversed by PI3K activator IGF-1. LY294002, a PI3K/Akt inhibitor, was used by concentration gradient before irradiation. Optical microscope showed the cells which treated with only radiation showed an elongated mesenchymal-like morphology and the cells treated with LY294002 keep their cuboidal appearance (**Figure 4C**). By western blot, we detected a dose-dependent increase of e-cadherin as well as a dose-dependent decrease of α-SMA and vimentin (**Figure 4D**). It indicated that inhibition of PI3K/Akt sig-

naling pathway can reduce EMT. PTEN is a known target of miR-21, and also it is an important suppressor gene of PI3K/Akt signaling pathway. We determined the expression of PTEN by western blot. As depicted in **Figure 4E**, the expression of PTEN decreased after irradiation, and the cells transfected with miR-21 inhibitor before irradiation were higher to express PTEN then the cells only treated with radiation. These results demonstrated that miR-21 inhibitor could up-regulate the expression of PTEN and then affect PI3K/Akt signaling pathway further reduce EMT.

Discussion

Radiotherapy is the common treatment for the patients with lung cancer. The Radiation-induced lung injury is inevitable and currently has no effective cure. New methods are needed for decrease this damage. Radiation has been shown to induce the alternation of many miRNAs expression. As a tumor gene, miR-21 has been shown to be a biomarker for the detection of various carcinomas [23]. Furthermore, it is also reported that miR-21 played a role in radiation and might be a potential biomarker in the breast cancer patients treated with radiotherapy [24]. In our study, we first demonstrated the potential involvement of miR-21 in radiation-induced injury in alveolar epithelial cells.

One of the major mechanisms of radiation-induced injury is epithelial-mesenchymal transition (EMT). To clarify the relationship between miRNAs and EMT will be benefit to solve the problem that radiation caused. Generally, EMT happens in embryonic development, most cancer cells and normal epithelial cells inflammation and fibrosis. Different kind of EMT has the similar mechanism which is characterized by a change of cell shape, cell immigration, and the loss of epithelial characteristics and the acquisition of mesenchymal phenotype [25]. The cell shape changes from cuboidal to fibroblastoid can be observed by microscope, and cell immigration can be examined by scratch assay to detect the progress of EMT. The other vital molecular feature of EMT is down-regulation of E-cadherin, which presents in the membrane of normal epithelial cells maintaining the cell polarity and morphological structure [26]. So, loss of E-cadherin disrupts the cell junction and changes the cell phenotype [27]. Meanwhile, vimentin and α-SMA are used to define the mesenchymal phenotype [28]. In our study, we first confirmed the radiation-induced EMT. We observed the cell shape by the optical microscope after treated with irradiation and found the EMT like change in phenotype. Then, we detected the expression changes of E-cadherin, α-SMA and vimentin in both gene and protein level by qPCR and Western blot analysis. And in scratch assay, we found that cells postirradiation were quicker to close a gap than the cells untreated. We concluded that radiation indeed induced EMT.

MiRNA played an important role in the process of EMT. Overexpression and inhibition of microRNAs have been demonstrated to be vital for cell properties and phenotypes. For example, miR-200s was explored for the cancer related EMT by targeting ZEB1 [29], and Let-7d was regarded as the member who took part in the fibrosis related EMT by targeting HMGA2 [30]. In this work, we explored the radiation-induced EMT through a single miRNA, miR-21. Following the radiation-induced EMT, the up-regulation of the miR-21 expression was found in RLE-6TN cells after irradiation. To investigate the relevance of radiation-induced EMT and miR-21, we transfected miR-21 inhibitor into suppress the miR-21 expression. Once we transfected miR-21 inhibitor before irradiation, the radiation-induced down-regulation of E-cadherin was reversed, as well as the changes of α-SMA and vimentin. And the same tendency was observed in cell type change through optical microscope and the cells immigration through scratch assay. These results revealed that miR-21 inhibitor could decrease the radiation-induced EMT. And in the other way round, the transfection of miR-21 mimic in normal RLE-6TN cells could enhance EMT.

There are many signaling pathways involved in EMT. Among these pathways, PI3K/Akt is a vital one. This signaling pathway is often studied in the progress of cancer, fibrosis and inflammation [31]. PTEN, which negatively regulated by miR-21, has been confirmed to be the validated target of miR-21 [32]. And PTEN is also a negative regulator of PI3K/Akt pathway activation, which has been involved in EMT [33]. We found that miR-21 negatively regulated EMT by inhibiting the activation of Akt. Studies have shown that TGF-β mediated EMT associated to PTEN/Akt signaling pathway [22]. In our work, PI3K

activator IGF-1 was used to prove this. We added IGF-1 after transfection miR-21 inhibitor before irradiation. The result showed that the expression of p-Akt got restoration. It means that miR-21 inhibitor attenuates the activation of Akt by PI3K. Afterwards, to expatiate this further, we used PI3K inhibitor LY294002 by concentration gradient before irradiation and detected the expression of E-cadherin, α-SMA and Vimentin. The western blot results of the tendency of these three critical proteins showed that the radiation-induced EMT recedes gradually according to concentration gradient. Then we detected the expressing of the miR-21 target protein PTEN. And an expected trend shows up. Taken together, miR-21 inhibitor attenuates radiation-induced EMT through PI3K/Akt by targeting PTEN.

In conclusion, we described the transfection of miR-21 inhibitor into RLE-6TN cells and examined its ability to cause changes in their phenotype and the expression of EMT relative protein and gene. We found transfecting miR-21 inhibitor into RLE-6TN cells before irradiation caused a less decrease in epithelial gene and protein expression levels, as well as a less increase in mesenchymal gene and protein levels, which accompanied changes in cellular phenotype and properties. It indicated that miR-21 inhibitor can attenuate the radiation-induced EMT. Furthermore, we have demonstrated the mechanism of this process and found that the activation of Akt decreased. It means that the PI3K/Akt signaling pathway involved in this process. And we confirmed this by using PI3K inhibitor LY294002. Then we determined the predicted target gene PTEN related to this process by western blot. These results suggested the miR-21 inhibitor had profound effects on reversing the radiation-induced EMT and may serve as a promising tool in increasing the potential therapeutic benefits of the lung cancer patient with radiation-induced lung injury.

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Disclosure of conflict of interest

None.

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