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Original Article

Anti-apoptotic roles of MicroRNA-21 via activating PTEN/PI3K/Akt pathway in lipopolysaccharide-induced acute lung injury

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Abstract: Acute lung injury (ALI) is a leading cause of death in critically ill patients, and apoptosis and inflammatory response are important pathological features of ALI. Recent studies showed that microRNAs (miRNAs) played important roles in a number of diverse biological and pathological processes, such as inflammation and apoptosis, but there is limited knowledge about their role in mediating ALI. Therefore, the aim of the present study is to evaluate the potential functions and mechanisms of miRNAs in ALI. ALI-relative miRNAs were retrieved from the Gene Expression Omnibus (GEO) under the accession number GSE18712 and the obtained miR-21 was validated by quantitative real-time PCR (qRT-PCR). The functional roles of miR-21 in LPS-challenged A549 cells were investigated, followed by bioinformatics methods and luciferase reporter assay to investigate whether PTEN was a direct target of miR-21. The effect of miR-21 on PI3K/AKT activation was also investigated by Western blotting. We found that miR-21 was significantly downregulated in lung tissues of ALI mice, and also decreased in LPS-challenged A549 cells. In addition, miR-21 overexpression restored the reduction of the cell viability induced by LPS and efficiently reduced LPS-induced apoptosis in A549 cells. Furthermore, phosphatase and tensin homologue (PTEN), which was a natural inhibitor of PI3K, was identified as a potential target of miR-21. miR-21 overexpression also exhibited a significant decrease in the expression levels of PTEN protein in A549 cells. Finally, the results from western blot analysis demonstrated that anti-apoptotic roles of miR-21 overexpression may be associated with activation of the PI3K/AKT pathway. Our results demonstrate that miR-21 plays an important role in LPS-induced apoptosis in mice and A549 cells, and that miR-21/PTEN/PI3K/Akt axis may become a novel therapeutic target for ALI.

Keywords: Acute lung injury, apoptosis, MicroRNA-21, PTEN/PI3K/AKT

Introduction

Acute lung injury (ALI) is a progressive syndrome with a high incidence and mortality rate, which is characterized by progressive and refractory hypoxemia and is the leading cause of death in critically ill patients [1]. The treatment of ALI remains one of the greatest challenges for the basic science and clinical investigators. Although new therapies have been appeared in clinic, all demonstrated limited efficacy thus far.

The pathophysiological mechanisms of ALI are complicated. Increasing evidence reveals that apoptosis plays an important role in different kinds of lung injury, including ALI [2-4]. Guinee et al. reported that apoptosis of epithelial cells was detected in diffuse alveolar damage, which is the predominant pathological finding in acute lung injury [5, 6]. It was also recently reported that apoptosis of epithelial cells and the Fas/FasL system have been implicated in the pathogenesis of ALI [7]. Inhibition of these signaling components can attenuate the severity of LPS-induced ALI in experimental animals [2, 8]. However, the specific mechanism of apoptosis within this context is extremely complicated and remains unclear.

MicroRNAs (miRNAs) are a class of endogenous small noncoding RNAs with 18-22 nt in length [9]. Mature miRNAs bind to the 3'-UTR of their target transcripts, and repress translation or degradation of the mRNAs [10]. Recent observations showed that miRNAs regulated many

biological processes, including cell proliferation, differentiation, metabolism and especially apoptosis [11]. And, kinds of miRNAs have been confirmed to participate in the development of ALI [12, 13]. Ke et al. found that miR-203 promoted apoptosis in LPS-stimulated alveolar epithelial cells by targeting PIK3CA [14]. Therefore, more extensive investigations on the identification and the functions of ALI relative miRNAs are required to elucidate the role of miRNAs in the pathogenesis of ALI.

In the present study, we reported that miR-21 was downregulated inlung tissues of ALI mice and miR-21 overexpression inhibited LPS-induced apoptosis by targeting PTEN via PI3K/AKT pathway. Our findings provided new insights into the molecular function of miR-21/PTEN/PI3K/AKT signaling pathway in the progression of ALI, suggesting that miR-21 can be used as a potential therapeutic target which may improve the treatment of ALI.

Materials and methods

Mice and preparation of ALI model

Studies were performed in male C57BL/6J mice (6-8 weeks old) that were purchased from the Department of Experimental Animals, Chongqing medical university. All animal procedures were conducted after approval from the Animal Committee of Yongchuan hospital of Chongqing medical university. ALI model was induced in mice as described previously [15, 16]. Briefly, ten mice were anesthetized with diethyl ether and then intravenously injected with 0.1 mg of lipopolysaccharide (LPS) (Sigma-Aldrich, St. Louis, MO, USA) in 25 μL of phosphate-buffered solution (PBS). Another 10 normal mice were used as controls. Lung tissues were harvested at 0 h, 6 h, 12 h and 24 h post-LPS challenge.

Cell culture and treatments

The human pulmonary epithelial cell line A549 was cultured in RPMI-1640 medium (Gibco, Carlsbad, CA, USA) containing 10% fetal bovine serum (FBS), 1% penicillin/streptomycin in a humidified incubator with 5% CO_2 at 37°C. The cells were seeded in six-well plates at 80% confluence. Cells were treated with 1 μg/ml LPS or vehicle (dimethyl sulfoxide; Sigma-Aldrich) for 24 h.

Choice of differentially expressed miRNAs list using heat map analysis

We obtained the microarray date from Gene Expression Omnibus (GEO, http://www.ncbi.nlm.nih.gov/geo/), and the GEO accession number is GSE18712. The date was generated using the genechip Affymetrix Human Genome U133 Plus 2.0 Array GPL570 (HG-U133_Plus_2), which completely coverage Human Genome U133 Set plus 6500 additional genes for analysis of over 47,000 transcripts.

Observations with adjusted *P*-values ≥ 0.05 were removed, and thus excluded from further analysis. The heat map of the 49 miRNAs most obvious differences was created using a method of hierarchical clustering by GeneSpring GX, version 7.3 (Agilent Technologies, California, United States).

Transfection

MiR-21 mimics and controls were purchased from Shanghai GenePharma (Shanghai, China). A549 cells were transfected with 20 nM miR-21 mimic or NC miRNAs with Lipofectamine™ RNAiMAX (Life Technologies, Grand Island, NY, USA). 48 hours after transfection, cells were collected for further protein extraction.

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

Total RNA of the cultured cells and the tissues was extracted using a mirVana miRNA isolation kit (Ambion, Carlsbad, CA, USA). miR-21 was reverse transcribed using the PrimeScript RT reagent Kit (TaKaRa, Tokyo, Japan) and quantified by real-time PCR with the TaqMan MicroRNA assay kit (Applied Biosystems). U6 was used for the normalization of miR-21 expression. All reactions were conducted in triplicate.

Cell viability assay

Cell viability was determined using the 3-(4, 5-Dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) assay (Amresco, Solon, USA) after 1 μg/ml LPS treatment. Briefly, A549 cells were seeded in 96-well culture plates with 1×10^4 cells/well, and incubated at 37°C with 5% CO_2. Then, 20 μl of MTT solution (5 mg/ml) was added to each well, and the cells were continuously incubated for 4 h. After incubation, 200 μl

of DMSO was added to each well to dissolve the formazan formed and the absorbance was read with a microplate reader (BioTek, Richmond, USA) at 490 nm according to the manufacturer's instructions.

Assessment of apoptosis

After treatment, A549 cells were suspended in binding buffer containing Annexin V-FITC and propidium iodide according to manufacturer's instructions (Invitrogen Life Technologies) and assessed by flow cytometry. At least 20,000 cells were acquired for each sample. The experiments were performed in triplicate.

Caspase 3 activity

For assessment of caspase-3 activity, A549 cells were treated with 10 ng/μl LPS in 0.5% FBS medium for 24 h. Caspase-3 activity was measured with the ApoAlert Caspase 3 Colorimetric Assay Kit (Clontech) according to the manufacturer's instructions.

Luciferase reporter assay

A whole fragment of 3'UTR PTEN-5 mRNA and a mutant form were cloned into pGL-3-Luc. The A549 cells were seeded in 12-well plates and co-transfected with pGL-3-PTEN-5 wild-type or mutant portion and TK100 Renilla combined with miR-21 mimic or NC control using Lipofectamine 2000 (Invitrogen). After 48 h of incubation, cells were collected for application in the Dual-Luciferase Reporter System (Promega, Madison, WI) following the manufacturer's recommendations. All of the dual-luciferase reporter assays were done in triplicate within each experiment, and three independent experiments were conducted.

Western blot

Total cellular proteins were extracted using RIPA lysis buffer containing proteinase inhibitor (Sigma, USA). Concentrations of total cellular protein were determined using a BCA assay kit (Pierce, Rockford, IL, USA). Total protein samples (40 μg) were analyzed by 8% SDS-PAGE gel and transferred to polyvinylidene difluoride (PVDF) membranes by a wet blotting procedure (100 V, 120 mins, 4°C). After blocked with 5% blocking buffer, the membranes incubated with primary antibodies against PTEN (1:500, Abcam, Cambridge, UK), Akt, phosphor-Akt (p-Akt), mTOR, phosphor-mTOR (p-mTOR), phosphor-p70S6K and p70S6K (1:2000, Cell Signaling Technology, MA, USA), cleaved caspase 3 (rabbit, 1:2000, Abcam, Cambridge, UK), at 4°C overnight. Control antibodies were anti-β-actin (1:2000; Santa, Santa Cruz, CA, USA). The protein bands were visualized by enhanced chemiluminescence detection reagents [17] (Applygen Technologies Inc., Beijing, China) as described in the manufacturer's instructions. Relative band intensities were determined by densitometry using Scion image software (version 4.0).

Statistical analysis

Statistical analyses were performed with SPSS 13.0 software. The results were evaluated by χ^2 test and the other data were evaluated by Student's t-test and expressed as the mean ± SD from three independent experiments. A *P*-value of less than 0.05 was considered statistically significant.

Results

miR-21 is significantly downregulated in lung tissues of ALI mice

Increasing evidences demonstrated that miRNAs play important roles in the progression of ALI. According to the heatmap obtained from GEO database under the accession number GSE18712; it was found that the expression levels of 49 ALI-relative miRNAs were dysregulated in the ALI group, including 25 down-regulated miRNAs and 24 up-regulated miRNAs (**Figure 1A**). It is known that apoptosis has a critical role during injury progression. For this reason, among the aberrantly expressed miRNAs, miR-21 was chosen as the candidate for further study because of its central role in lung injury [18]. Therefore, we next determined the expression levels of miR-21 using qRT-PCR. miR-21 was also downregulated in LPS-induced ALI mice (**Figure 1B**). Moreover, miR-21 expression was substantially downregulated in a time-dependent manner after LPS treatment (**Figure 1C**). These results suggest that miR-21 may play an important role in ALI.

miR-21 is involved in LPS-induced apoptosis in A549 cells

Given the limitations of primary cell culture, we used A549 cells stimulated by LPS to mimic ALI

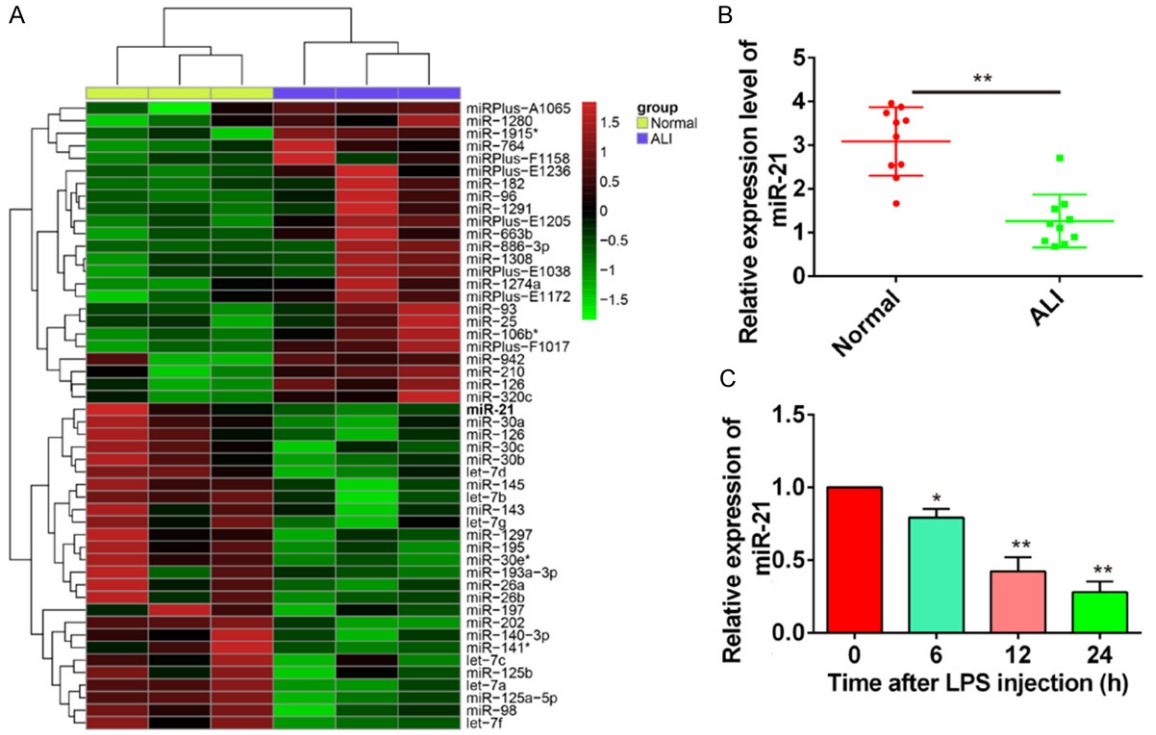

Figure 1. miR-21 is significantly downregulated in lung tissues of ALI mice. A. Differentially expressed miRNAs were analyzed using significance analysis of microarrays method. Data was retrieved from GEO dataset, with the accession number GSE18172. Expression values are represented in shades of red and green indicating expression above and below the median expression value across all samples. B. The expression of miR-21 was measured by qRT-PCR in LPS-induced mice (n=10). C. miR-21 expression was validated by qRT-PCR at 0 h, 6 h, 12 h and 24 h after LPS injection. **P < 0.01 vs the normal group. Data are the mean values ± SD, n = 10.

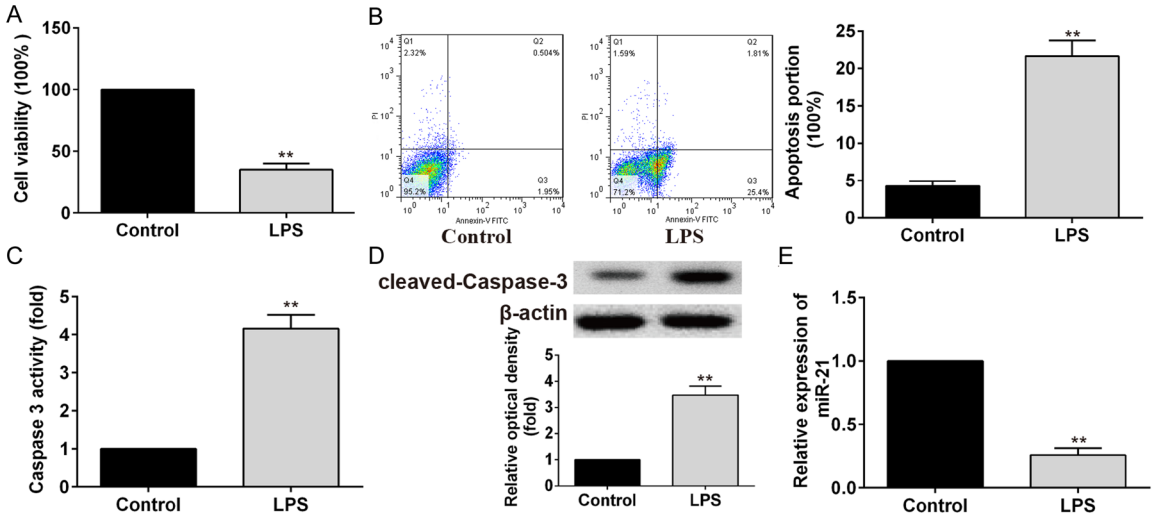

Figure 2. miR-21 is involved in LPS-induced apoptosis in A549 cells. A549 cells were exposed to LPS (1 μg/mL). A. A549 cell viability was measured by MTT assay. B. Cell apoptosis was performed by flowcytometry. C. Caspase-3 activity was measured with the ApoAlert Caspase 3 Colorimetric Assay Kit. D. Protein expression of cleaved caspase-3. β-actin was used as the internal control. E. The expression of miR-21 in A549 cells treated with LPS (1 μg/ml) for 24 h. Expression was normalized to U6. All data are expressed as the mean ± SD. **P < 0.01 vs the normal group.

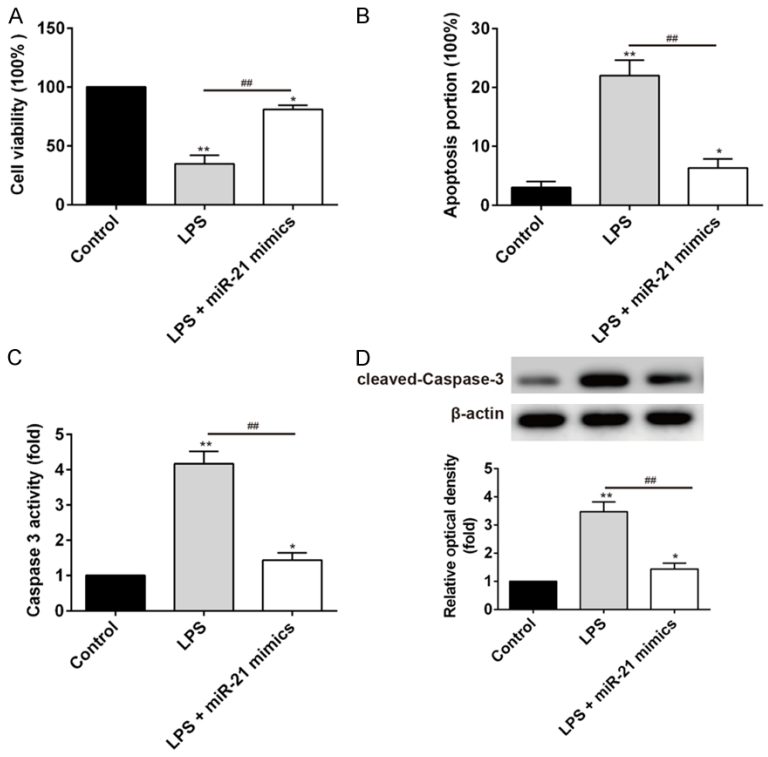

Figure 3. miR-21 mimic attenuates LPS-induced apoptosis in A549 cells. A549 cells were transfected with miR-21 mimic, then exposed to LPS. A. A549 cell viability was measured by MTT assay. B. Cell apoptosis was performed by FCM. C. Caspase-3 activity was measured with the ApoAlert Caspase 3 Colorimetric Assay Kit. D. Protein expression of cleaved caspase-3. β-actin was used as the internal control. All data are expressed as the mean ± SD. *P < 0.05, **P < 0.01 vs the normal group, ##P < 0.01 vs LPS + miR-21 mimic.

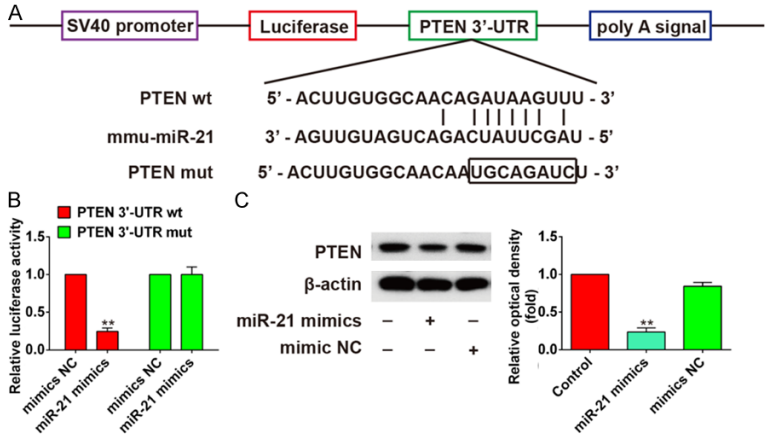

Figure 4. PTEN is a direct target of miR-21. A. Schema of the firefly luciferase reporter constructs for the PTEN, indicating the interaction sites between miR-21 and the 3'-UTRs of PTEN. B. Luciferase activities. A549 cells were co-transfected with firefly luciferase constructs containing the PTEN wild-type or mutated 3'-UTRs and miR-21 mimic or mimics NC, as indicated (n=6). C. Protein expression of PTEN after treatment with miR-21 mimic or mimics NC and the optical densities of the bands were measured using Image-Pro Plus software (n=6). All data are expressed as the mean ± SD. **P < 0.01 vs mimics NC.

in vitro as previously described reports [19]. After 1 μg/ml LPS treatment, we found that cell viability was significantly decreased in comparison with the control group (**Figure 2A**). Next, we examined effect of LPS treatment on apoptosis, which play a vital role in ALI. As shown in **Figure 2B**, the apoptosis of A549 after LPS treatment was markedly increased in comparison with the control group. In addition, this apoptosis was further confirmed by the caspase-3 activity assay (**Figure 2C**). Meanwhile, protein expression of cleaved (active form) caspase-3, a critical executioner of apoptosis, was measured by western blot. As shown in **Figure 2D**, the cleaved caspase-3 level was significantly increased when compared with control groups. To determine the potential involvement of miR-21 in LPS-induced apoptosis, we measured the expression levels of miR-21 after LPS treatment in A549 cells. Consistent with the results from the in vivo studies, miR-21 was significantly downregulated by stimulation with LPS when compared with the control group. Combined with previous studies, we hypothesized that miR-21 may be involved in LPS-induced apoptosis.

miR-21 mimic alleviates the apoptosis induced by LPS

To determine the regulatory role of miR-21 in LPS-induced apoptosis, A549 cells were transfected with 20 nM miR-21 mimic. As shown in **Figure 3A** and **3B**, overexpression of miR-21 significantly increased cell viability and decreased apoptosis after LPS treat-

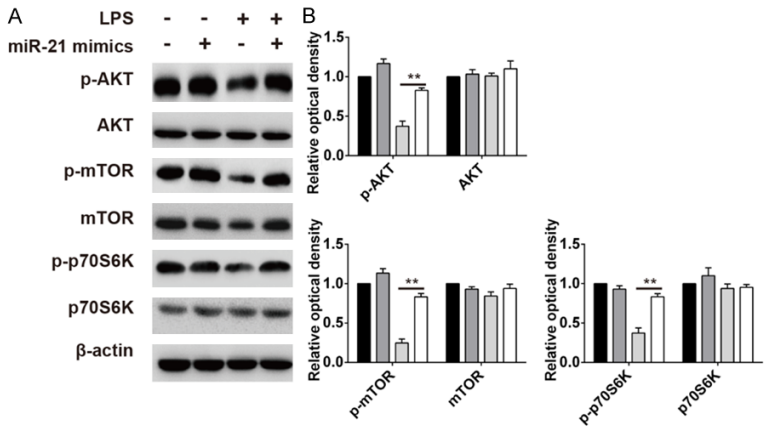

Figure 5. miR-21 mimic attenuated the A549 cells apoptosis induced by LPS via activation of PI3K/AKT pathway. A. Western blot showed changes of p-AKT, AKT, p-mTOR, mTOR, p-p70S6K and p70S6K protein levels in A549 cells transfected with miR-21 mimic, then exposed to LPS. B. The optical densities of the bands were measured using Image-Pro Plus software. All data are expressed as the mean ± SD. **P < 0.01 vs LPS group.

ment in comparison to the LPS group. Meanwhile, miR-21 decreased caspase-3 activity in comparison with the LPS group. More importantly, the level of cleaved caspase-3 was significantly decreased after miR-21 mimic transfection following LPS treatment. Taken together, these data suggest that miR-21 overexpression attenuates LPS-induced apoptosis in A549 cells.

PTEN is a target of miR-21

Bioinformatics analysis was performed in order to predict the target genes for miR-21. PTEN was predicted to be a candidate target of miR-21 (**Figure 4A**). To test the function of this potential binding site, we inserted wild-type or mutant 3'UTR sequences immediately downstream of the luciferase reporter gene and co-expressed these with either miR-21 mimic or miR-ctrl in A549 cells. As shown in **Figure 4B**, miR-21 overexpression caused a clear decrease in relative luciferase activity, whereas activity did not drop at all in the mutant 3'UTR reporter, indicating that functionality depends on the intact seed sequence. In addition, western Blot analysis showed that miR-21 overexpression decreased the levels of PTEN protein expression in A549 cells (**Figure 4C**). These results indicated that PTEN was a direct target of miR-21 in A549 cells.

miR-21 mimic attenuates LPS-induced apoptosis via activating PI3K/Akt/Mtor pathway

Recently studies have demonstrated that downregulation of PTEN was closely correlated with PI3K/AKT activation in several human malignancies [20, 21]. In addition, it has been reported that miR-21 activates the PI3K/Akt pathway through inhibiting the expression of PTEN in LPS-induced inflammatory lung injury [18], but this effect has not been investigated in ALI. Further studies were designed to explore the effects of miR-21 on activation of Akt in LPS-stimulated A549 cells. Western blot was performed to detect the protein levels of PI3K/Akt pathway in A549 cells transfected with miR-21 mimic, followed by LPS treatment. We confirmed that PI3K/AKT relativeprotein expressions, includingp-Akt, p-mTOR and p-p70-S6K were significantly decreased in the LPS group compared with control group, while the expressions of AKT, mTOR and p70S6K had no changes. However, enforced miR-21 expression effectively reversed the inhibitory effect of LPS on the expression levels of p-Akt, p-mTOR and p-p70S6K in A549 cells (**Figure 5A**, **5B**). Taken together, our results suggested that up-regulation of miR-21 could re-activate the PI3K/AKT pathway through downregulation of PTEN, finally attenuate the apoptosis induced by LPS.

Discussion

In the present study, we have identifiedthe central role of miR-21 in LPS-induced ALI. Our data showed that miR-21 was downregulated in LPS-induced ALI mice model. Furthermore, upregulation of miR-21 can inhibit PTEN expression and lead to an activation of PI3K/AKT pathway, finally protect A549 cells from apoptosis induced by LPS. These results indicate that miR-21 may be a potential therapeutic target in ALI.

Apoptosis is a very important pathological feature of ALI, and LPS has been used as a tool to study the mechanisms of ALI in animals [3, 22, 23]. Recently, more and more researches confirmed that microRNAs were involved in the regulation of proliferation and apoptosis of cancer cells [24, 25]. However, limited studies have paid much attention on the functions of miRNAs in ALI. To define the ALI-related miRNA pattern, we excavated a miRNA dataset (GSE-18172) that displayed a set of differentially reg-

ulated miRNAs, including miR-21, which was deregulated in lung tissues of ALI mice. Interestingly, our results showed that LPS reduced miR-21 expression in ALI animal model. Similar to the results from the lungs, A549 cells exposed to LPS displayed apoptosis and downregulated miR-21 levels. Previous researches demonstrated that there was a negative correlation between apoptosis and miR-21 expression [26], which suggested that miR-21 inhibition may aggravate apoptosis. In our study, we observed that treatment with the miR-21 mimic abolished the promoting effect of LPS on the apoptosis of A549 cells, along with decreased caspase-3 activity and cleaved caspase-3 expression. All these results indicated that miR-21 overexpression attenuated LPS-induced apoptosis in A549 cells.

MiRNAs post-transcriptionally regulate gene expression by inhibition of protein synthesis or/and degradation of target mRNA [11]. Several studies have proved that PTEN was one of miR-21's target genes in many cell types, such as hepatocytes, cardiomyocytes, and cancer cells [18, 27, 28]. For example, Liu MH et al. demonstrated that miRNA-21 knockdown in K562 cell could suppress the PI3K/AKT pathway by upregulation of PTEN expression [29]. Moreover, PI3K-Akt pathway activation is a downstream effect of PTEN inhibition, and the PI3K-Akt pathway represents a key mechanism during the ALI process [30]. Recently, Lai et al. found that suppression of PTEN could protect the lung parenchyma against acute injury in the oleic acid-ALI model [31]. Thus, we investigated whether upregulation of miR-21 attenuated apoptosis induced by LPS via targeting PTEN. As expected, our results showed that PTEN was identified as a target of miR-21 and upregulated miR-21 could inhibit PTEN protein expression in A549 cells. This finding partly agrees with the results reported by Liu MH et al. [29], suggesting that miR-21/PTEN/AKT pathway played an important role in the mechanism of LPS induced apoptosis in ALI.

Recently, many new insights into the core signaling pathways in the development process of ALI have been made, including ERK and Akt pathways [32]. A recent study reported that miR-203 accelerated apoptosis in LPS-stimulated alveolar epithelial cells through PI3K/Akt pathway [14]. Moreover, it has been reported that miR-21 affected the PI3K/Akt pathway by targeting PTEN [33], but this effect has not been investigated in LPS-induced ALI. In the present study, miR-21 mimic reversed the inhibitory effect of LPS on phosphorylation of Akt, mTOR and p70S6K, indicating that miR-21 may be an important regulator of PI3K/Akt signaling pathway in ALI.

In conclusion, our study provides evidence that miR-21 upregulation attenuates ALI-mediated apoptosis via targeting PTEN through activation of PI3K/AKT pathway. These results suggest that miR-21/PTEN/PI3K/AKT axis may be a therapeutic target for treatment of ALI.

Acknowledgements

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Disclosure of conflict of interest

None.

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