miR-146b-3p regulates PI3K/AKT signaling pathway in septic mice with acute respiratory distress syndrome

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Abstract

Background: This study aimed to explore the effect of miR-146b-3p on acute respiratory distress syndrome in septic mice by regulating PI3K/AKT signaling pathway.

Methods: Seventy C57BL/6 mice were divided into normal group (n = 10) and modeling group (n = 60, mice for constructing septic mice models with acute respiratory distress syndrome). Model mice were subdivided into model group (without any treatment), negative control (NC) mimic group (injection with miRNA NC), miR-146b-3p mimic group (injection with miR-146b-3p mimic), si-NC group (injection with PI3K γ siRNA NC), si-PI3K γ group (injection with PI3K γ interference sequence), and miR-146b-3p mimic + oe-PI3K γ group (injection with miR-146b-3p mimic + PI3K γ overexpression plasmid). Dual-luciferase reporter assay was conducted to determine the target relationship between miR-146b-3p and PI3K γ . Wet weight/dry weight (W/D) ratio of the left lung was measured. Hematoxylin and eosin stain was used to detect the pathological change of mouse lung. ELISA was employed to measure serum interleukin (IL) -1 β and IL-18 levels. miR-146b-3p and PI3K γ expressions were detected by qRT-PCR. PI3K γ , AKT, NLRP3, apoptosis-associated speck-like protein caspase recruitment domain (ASC) and Caspase-1 protein expressions were detected by Western blotting.

Results: miR-146b-3p negatively regulated PI3K γ . The lung tissues in other groups compared with normal group had down-regulated miR-146b-3p, up-regulated PI3K γ , p-AKT, ASC, NLRP3 and Caspase-1 proteins, higher W/D ratio, and more serum IL-1 β and IL-18 (all $P < 0.05$). All indicators in miR-146b-3p mimic group and si-PI3K γ group were significantly improved as compared to model group (all $P < 0.05$). Up-regulated PI3K γ , p-AKT, ASC, NLRP3 and Caspase-1 proteins and higher W/D ratio and IL-1 β and IL-18 levels in serum existed in miR-146b-3p mimic + oe-PI3K γ group compared with miR-146b-3p mimic group (all $P < 0.05$).

Conclusions: Up-regulation of miR-146b-3p can restrain PI3K/AKT signaling pathway, thereby improving acute respiratory distress syndrome in septic mice.

Background

Acute respiratory distress syndrome (ARDS) is a non-cardiogenic pulmonary edema caused by the accumulation of extravascular lung water as well as a major complication of severe sepsis and septic shock [1]. ARDS is characterized by hypoxemia, pulmonary edema, and significant respiratory failure over time, which leads to multiple organ failure and high mortality (up to 60%) [2–5]. Lipopolysaccharide (LPS) is the main method to construct sepsis-induced ARDS model, because it can activate inflammatory cells and release inflammatory factors interleukin-1 β (IL-1 β) and interleukin-18 (IL-18) [6]. NLRP3 inflammasome has vital function in the pathological process of ARDS. NLRP3 inflammasome as a protein complex contains pro-caspase-1 and apoptosis-associated speck-like protein caspase recruitment domain (ASC); active caspase-1 stimulates IL-1 β and IL-18, causing the release of these active cytokines [7].

As a complex pathological process, ARDS is regulated by transcription factors as well as miRNA. A miRNA is a small endogenous non-coding RNA molecule that includes approximately 18–24 nucleotides. It usually complements sites in the 3' untranslated region of the target messenger RNA for inhibiting the expression of post-transcriptional gene [8]. Several studies have found that multiple miRNAs affect the occurrence of ARDS, and microRNA-122 has relations with the mortality of ARDS and acute liver injury [9]; microRNA-211 induced by NF- κ B inhibits the function of macrophages releasing IL-10 in LPS-induced ARDS rats [10]. Down-regulation of microRNA-494 alleviates lung injuries in sepsis-related ARDS by regulating NQO1-Nrf2 signaling pathway [11]. microRNA-23a-5p can be used as a potential biomarker for early sepsis induced ARDS [12]. miR-146b mainly functions as a regulatory factor of inflammation and cancer [13]; reports about miR-146b are mostly related to cancer, but little is about its effect on ARDS [14]. AKT1 gene, known as protein kinase B α , is a key member in AKT family. Moreover, AKT is generally recognized as a key factor in PI3K (phosphatidylinositol 3-kinase) /AKT pathway and acts as a crucial role in cell differentiation, proliferation, metabolism, apoptosis, protein synthesis and transcription [11]. Previous studies have indicated that ARDS can be treated by the inhibition of PI3K/AKT signaling pathway [15–18].

A target relationship between miR-146b-3p and PI3K γ was found by bioinformatics prediction. We speculated that miR-146b-3p, by targeted down-regulation of PI3K γ expression, might repress PI3K/AKT signaling pathway, thus improving ARDS in septic mice.

Methods

Grouping and treating

Seventy two-week-old healthy male C57BL/6 mice of clean grade (purchased from the Laboratory Animal Center of Wenzhou Medical University), weighing 35 ± 5 g, were divided into normal group ($n = 10$) and modeling group ($n = 60$). Mice in modeling group were used to construct septic mice models with ARDS [19]. Mice were anesthetized by intraperitoneal administration of 50 mg/kg pentobarbital sodium before exposing their trachea and right internal jugular vein; 5 mg/kg LPS (escherichia coli 0111:B4) were dripped once into 50 μ L sterile phosphate buffered saline (PBS). A total of 3×10^7 PFU Ad-omentin or Ad- β gal was injected into the internal jugular vein of mice 3 days before installing the airway with LPS or gas (PBS). All mice in normal group survived, and 18 mice in modeling group died 24 h later. Model mice were subdivided into 6 groups: model group (without any treatment), negative control (NC) mimic group (injection with miRNA NC), miR-146b-3p mimic group (injection with miR-146b-3p mimic), si-NC group (injection with PI3K γ siRNA NC), si-PI3K γ group (injection with PI3K γ interference sequence), and miR-146b-3p mimic + oe-PI3K γ group (injection with miR-146b-3p mimic + PI3K γ overexpression plasmid). Sequences and plasmids (Suzhou Jima Gene Co. Ltd., China) of 100 ng were intraperitoneally injected in mice once on alternate days for 3 times. Mice in each group were anesthetized using 0.1 ml/10 g 0.3% pentobarbital sodium solution after treatment. Then the eyeball was removed for blood collection to make mice die due to excessive blood loss under anesthesia, and lung tissue specimens were reserved. The lung tissues and venous blood of 5 mice in each group were used for detection, and the remaining

tissues were stored in liquid nitrogen. This study was approved by the Ethics Committee of The First People's Hospital of Wenling.

Dual-luciferase reporter system

The binding site of miR-146b-3p and PI3K γ was analyzed by bioinformatics prediction website (www.targetscan.org). Next, the target relationship between miR-146b-3p and PI3K γ was verified by dual-luciferase reporter system. Dual-luciferase reporter gene vector of target gene PI3K and mutant at the binding site of miR-146b-3p and PI3K γ , PGL3-PI3K γ wt and PGL3-PI3K γ mut, were constructed, respectively. Rellina plasmid and the two reporter plasmids with miR-146b-3p plasmid and NC plasmid respectively were co-transfected into HEK293T cells. Dual-luciferase reporter assay was performed after 24 h cell transfection. Cells in each group were lysed and centrifuged at 12,000 rpm for 1 min. The precipitation was discarded and the supernatant was collected. Luciferase activity was measured according to the instruction of dual-luciferase reporter kit (Promega, USA). Lysed cell samples were pipetted into eppendorf tubes. The 100 μ L firefly luciferase working solution were added into every 10 μ L samples to determine firefly luciferase activity followed by the addition of 100 μ L ranilla luciferase working solution to determine ranilla luciferase activity. Relative luciferase activity = firefly luciferase activity / ranilla luciferase activity.

qRT-PCR

We extracted total RNA by using Trizol (Item number 16096020, Thermo Fisher Scientific, New York, USA; Item number B1802, Harbin HaiGene Co. Ltd., China). Taq Man MicroRNA Assays Reverse Transcription Primer (Thermo Scientific, USA) was used to synthesize cDNA. SYBR[®] Premix Ex Taq[™] II kit (Xingzhi Biotechnology Co. Ltd., China) was used to perform fluorescent quantitative PCR detection. The working solution included 25 μ L SYBR[®] Premix Ex Taq[™] II (2 $\times$), 2 μ L PCR forward primer, 2 μ L PCR reverse primer, 1 μ L ROX Reference Dye (50 $\times$), 4 μ L DNA templates and 16 μ L ddH₂O. Fluorescent quantitative PCR was carried out by using ABI PRISM[®] 7300 system (Prism[®] 7300, Shanghai Kunke Instrument Equipment Co. Ltd., China). The reaction condition was that 32 cycles of pre-denaturation at 95 $^{\circ}$ C for 10 min, denaturation at 95 $^{\circ}$ C for 15 s and annealing at 60 $^{\circ}$ C for 30 s followed by extending at 72 $^{\circ}$ C for 1 min. Δ Ct = Ct_(target gene) - Ct_(GAPDH); $\Delta\Delta$ Ct = Δ Ct_(experimental group) - Δ Ct_(control group). miR-146b-3p and PI3K γ took U6 and GAPDH as the internal references, respectively. $2^{-\Delta\Delta$ Ct} showed the relative expression level of a target gene. Primers were shown in Table 1.

Table 1
qRT-PCR primer sequence.

Gene	Sequence
miR-146b-3p	Forward: 5'-TGAGAACTGAATTCCATAGGCT-3'
	Reverse: 5'-GCACTGTCAGACCGAGACAAG-3'
U6	Forward: 5'-CTCGCTTCGGCAGCACATATACT-3'
	Reverse: 5'-ACGCTTCACGAATTTGCGTGTC-3'
PI3Ky	Forward: 5'-GACGAATTCAAATTATAACCTGGGAAATGGAG-3'
	Reverse: 5'-GCAAAGCTTCTTTTATGCCTGACATTTCTT-3'
GAPDH	Forward: 5'-CCAATGTGTCCGTCGTGGATCT-3'
	Reverse: 5'-GTTGAAGTCGCAGGAGACAACC-3'

Western blot

We extracted total protein by using RIPA lysis buffer containing PMSF (R0010, Solarbio). Protein concentration was determined using BCA kit (Thermo Fisher, USA), and the concentration was adjusted using deionized water. Samples were mixed with loading buffer and heated on the boiling water bath for 10 min. Protein samples of 30 µg were added into wells in each lane, and electrophoresis was conducted at a constant voltage of 80 V for 2 h. Proteins were transferred to PVDF membrane (ISEQ00010, Millipore, Billerica, MA, USA) at 110 V for 2 h. PVDF membrane was sealed with 5% skimmed milk powder at 4 °C for 2 h. The sealing solution was discarded, and then the membrane was washed with tris buffered saline tween (TBST) once. Primary antibodies rabbit anti-mouse PI3Ky (ab140307, 1:1,000, Abcam, UK), AKT (ab179463, 1:10,000, Abcam, UK), AKT (phospho S473) (ab81283, 1:5,000, Abcam, UK), NLRP3 (ab232401, 1:1,000, Abcam, UK), ASC (ab155970, 1:1,000, Abcam, UK), Caspase-1 (ab238979, 1:1,000, Abcam, UK) and GAPDH (ab8226, 1:2,000, Abcam, UK) were dropwise added in the membrane and incubated at 4 °C overnight. Then the membrane was washed with TBST three times, for 10 min per time. The membrane was incubated with the addition of horseradish peroxidase-labeled goat anti-rabbit IgG antibody (1:5,000, Beijing Zhongshan Biotechnology Co. Ltd., China). After rinsing with TBST, the membrane was placed on a clean glass plate. An equal amount of liquid A and liquid B in ECL fluorescence detection kit (Item Number BB-3501, Ameshame, UK) was mixed in a darkroom and dropwise added on the membrane. Then images were exposed by a gel imager. Images were taken using Bio-Rad image analysis system (BIO-RAD, USA) and analyzed using ImageJ software. Relative protein expression = gray value of the corresponding protein band / gray value of GAPDH protein band.

Wet weight/dry weight ratio

The left lung of the mouse was taken out by thoracotomy, and the blood on the lung surface was sucked dry by filter papers. Then wet weight was weighed out. After the left lung was baked in the oven at 80 °C for 48 h to achieve constant weight, the dry weight was weighed out. Wet weight/dry weight (W/D) ratio and lung water content were calculated to reflect the severity of pulmonary edema. $W/D \text{ ratio of the lung} = \text{wet weight of the lung} / \text{dry weight of the lung} \times 100\%$.

Hematoxylin and eosin stain

The lung tissue was fixed in 10% neutral formalin solution for 24 h, dehydrated in gradient alcohol and transparentized with xylene. Dried embedding salver was put in the paraffin cylinder. The tissue was embedded and sliced. Slices were transparentized with xylene, dehydrated in gradient alcohol and washed with distilled water for 1 min. Slices were stained with hematoxylin for 3 min and rinsed with running water. Then slices were differentiated in 0.5% hydrochloric acid alcohol differentiation solution for 10 s, blued by rinsing with running water for 10 min and stained in eosin solution for 5 min. Slices were dehydrated routinely, transparentized and sealed with neutral gum. Slices were observed under an optical microscope (XP-330, Shanghai Bingyu Optical Instrument Co. Ltd., Shanghai, China).

ELISA

Blood from the eyeballs of mice was placed at room temperature first and at 4 °C overnight and centrifuged at 3,500 xg. The supernatant was collected and cryopreserved at -80 °C. Levels of IL-1 β and IL-18 were detected by referring to the instructions of ELISA detection kits (IL-1 β and IL-18 detection kits number: 69-59812, 69-21183, Wuhan MSKBio, China).

Statistical analysis

All data were analyzed by SPSS21.0 statistical software. The measurement data were expressed as mean $\pm$ standard deviation. Comparison among groups was performed by one-way analysis of variance, and pairwise comparison of the mean among groups was carried out by Tukey post-hoc test. $P < 0.05$ indicated a statistically significant difference.

Results

miR-146b-3p targeted the negative regulation of PI3K γ gene

The specific binding site of miR-146b-3p and PI3K γ was verified by bioinformatics prediction website www.targetscan.org (http://www.targetscan.org/vert_72/) (Fig. 1a). Dual-luciferase reporter assay results showed that miR-146b-3p mimic group had lower luciferase activity for co-transfecting with wild type (Wt) -PI3K γ compared with NC mimic group ($P < 0.05$), and there was no significant difference in luciferase activity for co-transfecting with mutant (Mut) -PI3K γ plasmid ($P > 0.05$). Therefore, miR-146b-3p could target the negative regulation of PI3K gene (Fig. 1b).

miR-146b-3p, PI3K γ , AKT, p-AKT expressions in the lung tissue

We detected the expressions of lung tissue-related factors in each group to further confirm the regulation relationship between miR-146b-3p and PI3K/AKT signaling pathway (Fig. 2). The lung tissue in the rest groups compared with normal group had lower miR-146b-3p expression and higher PI3K γ expression (both $P < 0.05$). Compared with model group, NC mimic group, si-NC group, and miR-146b-3p mimic + oe-PI3K γ group had no significant differences in gene expressions ($P > 0.05$), and miR-146b-3p mimic group and si-PI3K γ group had lower PI3K γ and p-AKT protein expressions (both $P < 0.05$). miR-146b-3p mimic + oe-PI3K group had higher PI3K γ and p-AKT protein expressions as compared to miR-146b-3p mimic group (both $P < 0.05$).

Pathological change of mouse lung

Pathological change of mouse lung was detected by hematoxylin and eosin staining (Fig. 3). Mice in normal group had regular lung structure and no significant pathological injury. Mice in other groups had various degrees of inflammatory cell infiltration in the pulmonary alveoli and mesenchyme, effusion in the cavity, obvious thickening alveolar septum, partial collapsed alveoli, atelectasis, hyaline membrane, and damaged alveoli. Lung injuries in miR-146b-3p mimic group and si-PI3K γ group were significantly improved than model group, NC mimic group, si-NC group, and miR-146b-3p mimic + oe-PI3K γ group.

Wet weight/dry weight ratio of the lung

W/D ratio of the lung was significantly higher in other groups than normal group ($P < 0.05$). Compared with model group, NC mimic group, si-NC group, and miR-146b-3p mimic + oe-PI3K γ group had no difference in W/D ratio ($P > 0.05$), and W/D ratio was significantly lower in miR-146b-3p mimic group and si-PI3K γ group ($P < 0.05$). W/D ratio was significantly higher in miR-146b-3p mimic + oe-PI3K γ group than in miR-146b-3p mimic group ($P < 0.05$, Fig. 4).

Levels of IL-1 β and IL-18 in serum

Levels of IL-1 β and IL-18 in serum were detected by ELISA (Fig. 5). Levels of IL-1 β and IL-18 were significantly higher in other groups than in normal group (both $P < 0.05$). Compared with model group, NC mimic group, si-NC group, and miR-146b-3p mimic + oe-PI3K γ group had no difference in levels of IL-1 β and IL-18 (both $P > 0.05$), and miR-146b-3p mimic group and si-PI3K γ group had significantly lower levels of IL-1 β and IL-18 (both $P < 0.05$). Levels of IL-1 β and IL-18 were significantly higher in miR-146b-3p mimic + oe-PI3K γ group than in miR-146b-3p mimic group (both $P < 0.05$).

NLRP3, ASC and Caspase-1 protein expressions of the lung

NLRP3, ASC and Caspase-1 protein expressions were significantly higher in other groups than in normal group (all $P < 0.05$). Compared with model group, NC mimic group, si-NC group, and miR-146b-3p mimic + oe-PI3K γ group had no differences in NLRP3, ASC and Caspase-1 protein expressions (all $P > 0.05$), and miR-146b-3p mimic group and si-PI3K γ group had significantly lower NLRP3, ASC and Caspase-1 protein

expressions (all $P < 0.05$). NLRP3, ASC and Caspase-1 protein expressions were significantly higher in miR-146b-3p mimic + oe-PI3Kγ group than in miR-146b-3p mimic group (all $P < 0.05$, Fig. 6).

Discussion

Sepsis and acute respiratory distress syndrome (ARDS) are two types of syndromes needing intensive care with multiple characteristics. Both are common and highly lethal and have significant negative effects on survivors [20]. Sepsis-induced ARDS has an estimated incidence of over 40% and is the leading cause of ARDS death [21]. ARDS is a life-threatening complication featured by diffuse alveolar damage [22]. The incidence of ARDS varies widely, ranging from 1.5 patients per 100,000 people to nearly 79 patients per 100,000 people; European countries report lower incidence than the United States; mortality also varies due to the severity, age, and underlying medical conditions [23–27]. No monotherapy has been found to alter the latent pathological process of ARDS to date. The molecular mechanism for preventing the onset of ARDS is unclear and needs to be solved urgently [28].

In this study, down-regulated miR-146b-3p was found in sepsis-induced ARDS. Literatures revealed the activated PI3K/AKT signaling pathway in ARDS [16, 29]. In this study, we used PI3K interference sequence to treat C57BL/6 model mice and found that after PI3K/AKT signaling pathway was blocked, there were lower protein expressions of PI3Kγ, AKT, ASC, NLRP3 and Caspase-1, W/D ratio and serum IL-1β and IL-18 levels and improved inflammation in the lung tissues of mice. The results showed that inhibition of PI3K/AKT signaling pathway could improve lung injuries caused by ARDS in septic mice. To further explore the upstream regulatory mechanism of PI3K/AKT signaling pathway, we verified the target relationship between PI3Kγ and miR-146b-3p through bioinformatics prediction website and confirmed through dual-luciferase reporter assay that miR-146b-3p negatively regulated PI3Kγ. Model mice were injected with miR-146b-3p inhibitor + oe-PI3Kγ and miR-146b-3p mimic. The results indicated that miR-146b-3p overexpression improved lung injuries caused by ARDS in septic mice. miR-146b-3p inhibited PI3K/AKT signaling pathway by targetedly inhibiting PI3Kγ gene expression, thereby down-regulating the expressions of inflammasome ASC, NLRP3 and Caspase-1, improving inflammation, and reducing IL-1β and IL-18 levels in serum. Moreover, re-overexpression of PI3K restored the protection of miR-146b-3p overexpression on lung injury. The down-regulation of miR-146b-3p expression was detected in model mice in this study. The up-regulation of miR-146b-3p inhibited PI3Kγ expression, thereby inhibiting PI3K/AKT signaling pathway, down-regulating the protein expressions of inflammasome ASC, NLRP3 and Caspase-1, reducing serum IL-18 and IL-1β levels, and improving tissue inflammation, which was consistent with previous reports on ARDS in septic mice [30–34].

Conclusions

In this study, we demonstrate that miR-146b-3p, by targeting PI3Kγ gene, mediates PI3K signaling pathway to inhibit the recovery of lung injury in model mice. We further clarify the development mechanism of ARDS in septic mice, which lays a theoretical foundation for the clinical treatment of ARDS in septic mice. However, the association between miR-146b-3p and ARDS in septic mice has not

been fully explained to date; the molecular mechanism by which PI3K/AKT signaling pathway regulates inflammasome NLRP3, ASC and Caspase-1 has not been fully explored; the targeted regulatory network of miR-146b-3p in sepsis-induced ARDS mice is still unclear.

Abbreviations

ARDS: Acute respiratory distress syndrome; LPS: Lipopolysaccharide; IL: interleukin; NC: negative control; W/D: Wet weight/dry weight; ASC: apoptosis-associated speck-like protein caspase recruitment domain; PBS: phosphate buffered saline; TBST: tris buffered saline tween; Mut: mutant; Wt: wild type; 3'-UTR: 3' untranslated region.

Declarations

Ethics approval

This study was approved by the Ethics Committee of The First People's Hospital of Wenling.

Consent for publication

Not applicable

Availability of data and material:

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

Competing interests

The authors declare that they have no competing interests.

Funding

Not applicable

Authors' contributions

JQZ and XHJ contributed to data collection and data statistics. MPD collected data. YL and JFZ contributed to data analysis, manuscript concept and editing, and manuscript guidance. All authors read and approved the final manuscript.

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Not applicable

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Figures

Figure 1

miR-146b-3p inhibited PI3K/AKT signaling pathway in the lung tissue. a Sequence in 3'-UTR region of the binding site of miR-146b-3p and PI3K γ . b Luciferase activity. Compared with NC mimic group, *P < 0.05. wt wild type, mut mutant, NC negative control, 3'-UTR 3' untranslated region

Figure 2

miR-146b-3p inhibited Notch signaling pathway in the lung tissue. a Expression levels of miR-146b-3p and PI3K γ . b Protein bands of PI3K γ , AKT and p-AKT. c Protein expressions of PI3K γ , AKT and p-AKT. Compared with normal group, *P < 0.05; compared with model group, #P < 0.05; compared with NC mimic group, %P < 0.05; compared with miR-146b-3p mimic group, &P < 0.05; compared with si-NC group, \$P < 0.05; compared with si-PI3K γ group, @P < 0.05. NC negative control

Figure 3

Pathological change of mouse lung (200×). a Normal group. b Modeling group. c NC mimic group. d miR-146b-3p mimic group. e si-NC group. f si-PI3K γ group. g miR-146b-3p mimic + oe-PI3K γ group. NC negative control

Figure 4

Wet weight/dry weight ratio of the lung. Compared with normal group, * $P < 0.05$; compared with model group, # $P < 0.05$; compared with NC mimic group, % $P < 0.05$; compared with miR-146b-3p mimic group, & $P < 0.05$; compared with si-NC group, \$ $P < 0.05$; compared with si-PI3Ky group, @ $P < 0.05$. W/D Wet weight/dry weight, NC negative control

Figure 5

Levels of IL-1 β and IL-18 in serum. a Level of IL-1 β . b Level of IL-18. Compared with normal group, * $P < 0.05$; compared with model group, # $P < 0.05$; compared with NC mimic group, % $P < 0.05$; compared with miR-146b-3p mimic group, & $P < 0.05$; compared with si-NC group, \$ $P < 0.05$; compared with si-PI3Ky group, @ $P < 0.05$. IL interleukin, NC negative control

Figure 6

NLRP3, ASC and Caspase-1 protein expressions of the lung. a Protein bands of NLRP3, ASC and Caspase-1. b NLRP3, ASC and Caspase-1 protein expressions. Compared with normal group, * $P < 0.05$; compared with model group, # $P < 0.05$; compared with NC mimic group, % $P < 0.05$; compared with miR-146b-3p mimic group, & $P < 0.05$; compared with si-NC group, \$ $P < 0.05$; compared with si-PI3K γ group, @ $P < 0.05$. ASC apoptosis-associated speck-like protein caspase recruitment domain, NC negative control

Supplementary Files

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