Reduced peripheral blood miR-140 may be a biomarker for acute lung injury by targeting Toll-like receptor 4 (TLR4)

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Abstract. Acute lung injury (ALI) is a common complication of sepsis to which patients often succumb due to poor effective pharmacological interventions. Recent studies have focused on the potential application of circulating microRNAs (miRs or miRNAs) as novel prognostic and therapeutic biomarkers. The present study focuses mainly on miR-140, the role of which is poorly understood in the progression of ALI. The results of the present study revealed that toll-like receptor 4 (TLR4) expression was upregulated the lungs of rats with ALI. Meanwhile, serum levels of tumor necrosis factor-α, interleukin (IL)-6 and IL-1β were significantly increased in rats with ALI compared with normal control rats. These data indicated the successful establishment of LPS-induced ALI. Furthermore, miR-140 was decreased in the peripheral blood of patients with ALI compared with control subjects. Receiver operator characteristic analysis indicated that miR-140 could be used to screen ALI patients and distinguish them from healthy controls. MiR-140 was demonstrated to be downregulated in the plasma and lungs of rats with ALI compared with the normal control group. A dual luciferase reporter assay indicated that TLR4 was a target gene of miR-140. To investigate whether miR-140 exerted its role via TLR4, a specific TLR4-targeting small interfering RNA was selected. It was revealed that TLR4 silencing was able to suppress the phosphorylation of NF-κB even in cells transfected with miR-140 inhibitor. In summary, reduced miR-140 expression and increased TLR4 signaling activation may serve a key role in the progression of ALI.

Introduction

Acute lung injury (ALI) and the more severe form of ALI, acute respiratory distress syndrome (ARDS), typically have high morbidity and mortality (1,2). Lipopolysaccharide (LPS) derived from Gram-negative bacteria is a key pathogenic factor in the progression of ALI, (1,2). It has been reported that LPS induces the inflammatory response by binding LPS receptors and accessory proteins, thereby promoting the production of pro-inflammatory mediators (1-4). A number of studies have identified the important role of Toll-like receptor 4 (TLR4) in LPS-induced inflammation (5-7). TLR4 has been reported to activate the nuclear factor (NF)-κB signaling pathway, causing the transcription of pro-inflammatory cytokines, including tumor necrosis factor (TNF)-α (8).

MicroRNAs (miRs or miRNAs) are a class of small non-coding RNAs ~21-25 nucleotides in length that are found in almost all genomes (9,10). It has been suggested that miRNAs repress gene expression at the post-transcriptional level by binding to the 3' untranslated regions (3'UTRs) (11). Abnormal miRNA expression has been reported in a number of diseases, including cystic fibrosis, asthma, ALI and ARDS (12,13). MiR-34a levels are significantly higher in the lungs of neonatal rats exposed to hyperoxia compared with normal controls and miR-34a suppression improves the pulmonary phenotype and bronchopulmonary dysplasia-associated pulmonary arterial hypertension (14). Furthermore, plasma miR-200c-3p levels are much higher in patients with severe pneumonia compared with healthy controls (15).

MiRNAs have been detected in a variety of sources, including tissues, blood and body fluids (16,17). They are stable and resistant to different sample handling conditions and, as such, may have potential as biomarkers for the progression of a number of diseases (18,19). Recently, miRNAs have been demonstrated to be potential biomarkers for cancer as well as cardiovascular and rheumatic diseases (17-19). Plasma miR-155 and miR-146a may be used as novel biomarkers to predict the mortality and treatment outcome of severe sepsis and sepsis-induced acute lung injury (20).

MiR-140 has been reported to be aberrantly expressed in a number of tumor types, including glioma and gastric cancer (21,22). However, the specific role of miR-140 in the progression of ALI has never been explored. The aim of the present study was to measure the expression of miR-140 and assess the underlying mechanism by which it affects the progression of ALI.

Materials and methods

Study population. Briefly, 50 mechanically ventilated patients with ALI (mean age, 48.57±16.55 years; sex ratio, male:female,

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26:24; comorbidities, none) admitted to the Zhongnan Hospital of Wuhan University (Wuhan, China) were recruited from September 2016 to March 2017. A total of 20 healthy subjects (mean age: 45.25±10.24 years; sex ratio, 1:1; comorbidities, none) were recruited from September 2016 to March 2017 and screened at the same hospital. Screening consisted of history, physical examination, routine blood investigation, electro-cardiogram and spirometry. At the baseline, the mean forced expiratory volume and forced vital capacity were 4.2 (0.5 l) and 5.2 (0.75 l), respectively. Patients were included if they met the criteria for ALI, without sepsis (ALI criteria was established in accordance with the definition and diagnostic criteria of the ARDS Berlin in 2012). Upon admission to hospital, all patients, within the onset of 24 h, accepted oral tracheal intubation with ventilator-assisted respiration. The right subclavian vein catheter was also retained. Patients were excluded from the present study if they exhibited thoracic deformity, pneumothorax, severe myocardial ischemia, intracranial hypertension, cerebral blood supply insufficiency, hemodynamic instability or chronic organ failure. Patients were also excluded if they were <18 years of age. The study was approved by the local research ethics committee of Zhongnan Hospital of Wuhan University (Wuhan, China) and written informed consent was obtained from the legal representative of each patient or the subjects themselves in the control group. The research was carried out in compliance with the Declaration of Helsinki.

Animals. Male Wistar rats were supplied by SPF (Beijing) Biotechnology Co., Ltd. (Beijing, China). Rats were housed at 23±2℃ with a 12-h light/dark cycle in an air-conditioned room with 50±10% relative humidity. A normal rat pellet diet and water were supplied *ad libitum*. Rats were randomly allocated to different experimental groups. All animal procedures were performed in accordance with the Guide for the Care and Use of Laboratory Animals of Zhongnan Hospital of Wuhan University (23). Ethical approval was granted by the local ethics committee of Zhongnan Hospital of Wuhan University (Wuhan, China).

LPS (*Escherichia coli* serotype K235) were purchased from Sigma-Aldrich (Merck KGaA, Darmstadt, Germany). Rats were randomly divided into the control and LPS groups (n=6 in each group). Rats in the control group received saline (0.5 ml) intraperitoneally (IP). Rats in the LPS group received LPS (0.5 mg/kg, IP). Rats were anesthetized with ketamine + xylazine 20 h later, following which blood was sampled and lung tissues were harvested. Tissues were flash frozen in liquid nitrogen and stored at -70℃ for use in the myeloperoxidase assay, reverse transcription-quantitative polymerase chain reaction (RT-qPCR) and western blotting.

Histological assessment. Lung tissue samples were fixed in 4% phosphate-buffered neutral formalin at room temperature for 20 min, embedded in paraffin and cut into 5 µm thick sections. Samples were then deparaffinized, rehydrated using a descending alcohol series and microwave-heated for 30 min in sodium citrate buffer (Beijing Solarbio Science & Technology, Co., Ltd., Beijing, China) at 100℃ for antigen retrieval. Sections were subsequently incubated with 0.3% hydrogen peroxide/phosphate-buffered saline at room temperature for 30 min. Samples were incubated with hematoxylin (Beijing

Solarbio Science & Technology, Co., Ltd.) at room temperature for 5 min. Then, the slides were washed with ddH₂O for 3 min and further stained with eosin for 2 min at room temperature. Samples were washed a second time with ddH₂O for 3 min. Slides were visualized using light microscopy (magnification, x40; Olympus CK40, Olympus Corporation, Tokyo, Japan).

Cell culture. A549 human lung adenocarcinoma epithelial cells were purchased from the American Type Culture Collection (Manassas, VA, USA) and cultured in Ham's F12 nutrient medium (Hyclone; GE Healthcare Life Sciences, Logan, UT, USA). 293T cells were purchased from the Peking Union Medical College Cell Culture Center (Beijing, China) and cultured in Dulbecco's modified Eagle's medium (Hyclone; GE Healthcare Life Sciences). THP1 cells (American Type Culture Collection, Manassas, VA, USA) were cultured in RPMI-1640 medium (Hyclone; GE Healthcare Life Sciences). Cells were cultured in the appropriate medium supplemented with 10% fetal bovine serum (FBS; Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA), 100 U ml⁻¹ penicillin and 100 U ml⁻¹ streptomycin at 37℃.

RNA extraction. Total RNA was isolated from the whole blood samples (5 ml, collected in tubes containing EDTA) or epithelial cells using RNAVzol LS (Vigorous Biotechnology Beijing Co., Ltd., Beijing, China) according to the manufacturer's protocol. The concentration and the purity of RNA samples were determined by measuring the optical density (OD) 260/OD280.

RT-qPCR. A total of 1 µg RNA was reverse transcribed using Moloney Murine Leukemia Virus (MMLV) reverse transcription enzyme (Applied Biosystems; Thermo Fisher Scientific, Inc.) with specific primers. The temperature protocol used for RT was as follows 72℃ for 10 min; 42℃ for 60 min, 72℃ for 5 min and 95℃ for 2 min. To quantify the relative mRNA levels, qPCR was performed using SYBR Green Supermix (Bio-Rad Laboratories, Inc., Hercules, CA, USA) in an iCycleriQ real-time PCR detection system. The PCR amplifications were performed in a 10 µl reaction system containing 5 µl SYBR Green Supermix, 0.4 µl forward primer, 0.4 µl reverse primer, 2.2 µl double distilled H₂O and 2 µl template cDNA. Thermocycling conditions were as follows: 95℃ for 10 min followed by 40 cycles of 95℃ for 15 sec and 60℃ for 1 min. Relative mRNA expression was normalized to U6 using the $2^{-\Delta\Delta Cq}$ method (24). Primer sequences are as follows: miR-140-5p-RT, 5'-GTCGTATCCAGTGCAGGGTCC GAGGTATTCGCACTGGATACGACCTACCA-3'; U6-RT, 5'-GTCGTATCCAGTGCAGGG TCCGAGGTATTCGCACTG GATACGACAAAATG-3'; miR-140-5p, forward 5'-GCGCGC AGUGGUUUUACCCUA-3'; U6, forward 5'-GCGCGTCGT GAAGCGTTC-3'; universal reverse primer, 5'-GTGCAGGGT CCGAGGT-3'.

Western blotting. Total proteins were isolated from lung tissues or A549 cells using a total protein extraction kit (Beijing Solarbio Science & Technology Co., Ltd.) and collected following centrifugation at 12,000 x g for 30 min at 4℃. A BCA protein assay kit (Pierce; Thermo Fisher Scientific, Inc.) was used to determine the protein concentration. A total of 20 µg protein was separated using 12% SDS-PAGE, transferred

onto polyvinylidene difluoride (PVDF) membranes and blocked with 5% fat-free milk at room temperature for 2 h. Membranes were incubated with primary antibodies against TLR4 (cat. no. ab22048; 1:1,000; Abcam, Cambridge, UK), phosphorylated (p)-nuclear factor (NF)-κB (cat. no. 3033; 1:1,000; Cell Signaling Technology, Inc., Danvers, MA, USA), NF-κB (cat. no. 8242; 1:1,000; Cell Signaling Technology, Inc.) and anti-GAPDH (2118; 1:5,000; Cell Signaling Technology, Inc.) at 4°C overnight. Membranes were subsequently incubated with horseradish peroxidase (HRP)-conjugated goat anti-rabbit IgG (both 1:5,000; cat. no. ZB-2301; Beijing Zhongshan Golden Bridge Biotechnology Co., Beijing, China) for 2 h at room temperature, followed by three washes with TBST. Enhanced chemiluminescence (EMD Millipore, Billerica, MA, USA) was used to determine the protein concentrations according to the manufacturer's protocol. Signals were detected using a Super ECL Plus Kit (Nanjing KeyGen Biotech Co., Ltd.) and quantitative analysis was performed using UVP software (UVP LLC, Upland, CA, USA). Relative protein expressions were normalized to GAPDH. All experiments were repeated three times. ImageJ 1.43b software (National Institutes of Health, Bethesda, MD, USA) was used for densitometry analysis.

Transfection. MiR-140 mimics, inhibitors, TLR4 siRNA were obtained from Guangzhou RiboBio Co., Ltd. (Guangzhou, China) and transfected into A549 and 293T cells was performed using Lipofectamine 2000 (Invitrogen; Thermo Fisher Scientific, Inc.) according to manufacturer's protocol. Cells were collected for subsequent experimentation following the 48 h transfection.

ELISA. Serum or cell lysates were homogenized in lysis buffer (50 mmol/l Tris-HCl; 300 mmol/l NaCl; 5 mmol/l EDTA; 1% Triton X-100; and 0.02% sodium azide) containing a protease inhibitor cocktail (Roche Diagnostics, Basel, Switzerland) and then centrifuged at 16,000 x g for 15 min at 4°C. Levels of TNF-α (cat. no. DTA00D), interleukin (IL)-6 (cat. no. D6050) and IL-1β (cat. no. 201-LB) in the supernatant were measured using ELISA (all R&D Systems, Inc., Minneapolis, MN, USA) according to the manufacturer's protocol. Samples were read at a 450 nm using a microplate reader (Thermo Fisher Scientific, Inc.).

Luciferase reporter assay. The TLR4 3'UTR segments containing the miR-140 binding sites were amplified using PCR. To amplify TLR3 3'UTR, the genome DNA from A549 cells were extracted using an EasyPure Genomic DNA kit (cat. no. EE101-02, Beijing TransGen Biotech Co., Ltd., Beijing, China). PCR was performed using a 2xEasyTaq PCR SuperMix kit (cat. no. AS111-02; Beijing TransGen Biotech Co., Ltd.). The DNA polymerase used was part of this kit. 2 μl genome DNA (2 μl), 1 μl forward primer, 1 μl reverse primer, 25 μl 2xEasyTaq PCR SuperMix, and 20 μl nuclease-free water were mixed. The thermocycling conditions were as follows: 94°C for 5 min, followed by 40 cycles of 94°C for 30 sec, 55°C for 30 sec and 72°C for 30 sec, and then 72°C 10 min. The primers used were as follows: TLR4-3'UTR forward, 5'-GGC TCCTGATGCAAGATGCCCCT-3' and reverse, 5'-CTGCCT TGAATACCTTCACACGT-3'; GAPDH forward, 5'-CTGAGC ACCAGGTGGTCTC-3' and GAPDH reverse, 5'-CATGAC

AAGGTGCGGCTCC-3'. Oligonucleotides were then inserted into a pmirGLO Dual-Luciferase miRNA Target Expression Vector (Promega Corp., Madison, WI, USA) and sequenced to confirm there were no mutations. 293 cells were seeded in 24-well plates and co-transfected with miR-140 mimics or negative control miR with recombinant pmirGLO. The relative Luciferase activity was measured using the Dual-Luciferase Reporter Assay System (Promega Corp.) following 48 h co-transfection.

Statistical analysis. Data are presented as the mean ± standard deviation. Two-tailed unpaired student's t-tests were used for comparisons between two groups. Analysis of variance followed by Tukey's post hoc test were used for multiple group comparisons with SPSS 13 (SPSS, Inc., Chicago, IL, USA). Receiver operator characteristic (ROC) curves were used to assess the potential of miR-140 as a biomarker, and the area under curve (AUC) was recorded. $P<0.05$ was considered to indicate a statistically significant difference.

Results

Peripheral blood miR-140 was lower in patients with ALI. As shown in Fig. 1A, peripheral blood miR-140 was much lower in patients with ALI compared with healthy controls. In addition, ROC analysis revealed that peripheral blood miR-140 could be used to differentiate subjects with ALI from healthy controls, with an ROC curve area of 0.935 (95% confidence interval: 0.817-1.000; $P<0.0001$; Fig. 1B).

Serum levels of inflammatory factors are higher in patients with ALI. The serum levels of inflammatory factors, including TNF-α, IL-6 and IL-1β were measured. Compared with the healthy controls, serum TNF-α, IL-6 and IL-1β levels were significantly increased in patients with ALI (Fig. 2).

MiR-140 was lower in the peripheral blood and lung tissues of rats with LPS-induced ALI. LPS-induced ALI rat models were established. Real time PCR analysis indicated that miR-140 expression was decreased in the peripheral blood of rats with LPS-induced ALI compared with control rats (Fig. 3A). H&E staining revealed that rats in the LPS group developed lung inflammation, hemorrhaging and alveolar septal thickening compared with the normal control rats (Fig. 3B). In addition, the expression of miR-140 was decreased in the lung tissues of rats with ALI compared with control rats (Fig. 3C). ELISA results indicated that serum TNF-α, IL-6 and IL-1β were significantly upregulated in rats with ALI compared with control rats (Fig. 3D). These findings suggest a potential correlation between miR-140 and ALI-associated inflammatory responses.

TLR4 is a target gene of miR-140. To explore the potential mechanism by which miR-140 regulates the progression of ALI, potential target genes of miR-140 were searched using TargetScan (http://www.targetscan.org/vert_71/). Interestingly, a conserved binding site of miR-140 was identified on the 3'UTR of TLR4 (Fig. 4A). This 3'UTR region was cloned into pmirGLO plasmids. A dual luciferase reporter assay suggested that miR-140 significantly suppressed the relative luciferase activity of pmirGLO-TLR4-3'UTR (Fig. 4B), suggesting that
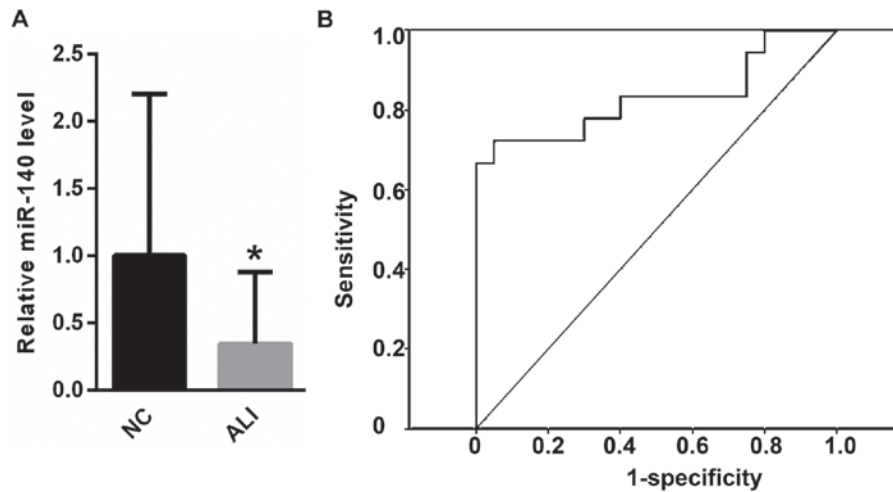
Figure 1. Peripheral blood miR-140 was lower in patients with ALI. (A) Peripheral blood miR-140 in patients with ALI and NCs. (B) ROC analysis of peripheral blood miR-140. *P<0.05 vs. NC. miR, microRNA; ALI, acute lung injury; NC, negative control subjects; ROC, receiver operator characteristic.
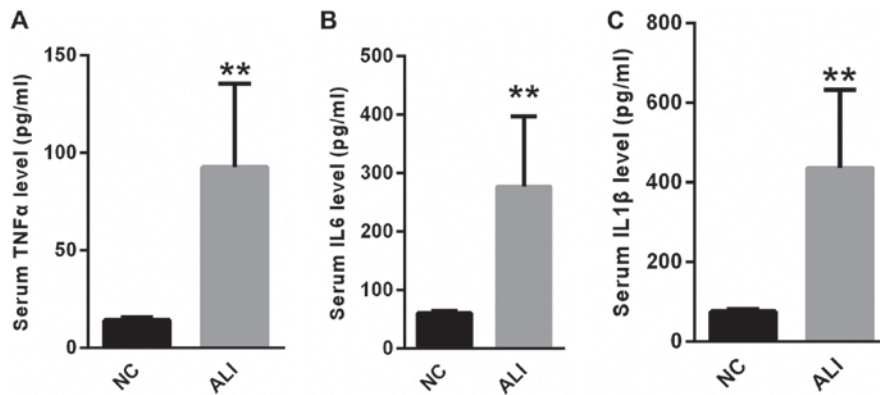

Figure 2. Serum levels of inflammatory factors were higher in patients with ALI. Compared with the NC group, serum (A) TNF-α, (B) IL-6 and (C) IL-1β levels were significantly increased in patients with ALI. **P<0.01 vs. NC. ALI, acute lung injury; NC, negative control subjects; TNF, tumor necrosis factor; IL, interleukin.

TLR4 is a target gene of miR-140. Furthermore, western blotting results revealed that TLR4 expression and NF-κB phosphorylation were much higher in the lung tissues of rats with ALI (Fig. 4C), suggesting a negative correlation between miR-140 and TLR4 expression.

TLR4 knockdown could reverse miR-140 inhibition-induced inflammatory response. To determine the role of TLR4 in the miR-140-induced inflammatory response, a specific siRNA targeting TLR4 was selected. As shown in Fig. 5, miR-140 inhibition increased the expression of TLR4 and enhanced the phosphorylation of NF-κB. In contrast, TLR4 silencing could suppress NF-κB phosphorylation in A549 cells transfected with miR-140 inhibitor (Fig. 5). These data suggest that the reduction of the miR-140-induced inflammatory response was mainly achieved through targeting TLR4.

Discussion

ALI is a common complication of sepsis, which often results in mortality due to a lack of effective pharmacological interventions (25,26). It is therefore important to explore effective prognosis prediction and treatment strategies for this disease (27). It has been widely reported that TLRs serve important roles in the progression of lung diseases (26). Microbe-derived LPS is suggested to be a major element involved in the development of sepsis (28). In general, LPS triggers inflammatory responses by activating TLRs, which then recruit neutrophils to the inflammatory site (25,29). However, when the defensive reactions are pathologically over-stimulated, acute hyper-inflammation can be induced and further exacerbates injury in the lung (30).

In inflamed tissues, TLR4 overexpression promotes neutrophil migration into the LPS-stimulated lungs (30,31). TLR4 interacts with the adaptor protein MYD88 and activates NF-κB signaling, thereby inducing TNF-α production in LPS-induced ALI (26). In line with previous studies, the results of the present study demonstrate that TLR4 expression is increased in the lungs of rats treated with LPS. Meanwhile, serum levels of TNF-α, IL-6 and IL-1β were significantly increased in LPS-treated rats compared with normal controls. These data indicated that LPS-induced ALI rat models had been successfully established.

A number of studies have reported that changes in miRNA expression are correlated with the immune response and inflammatory lung diseases, including ALI (12,32). In the past
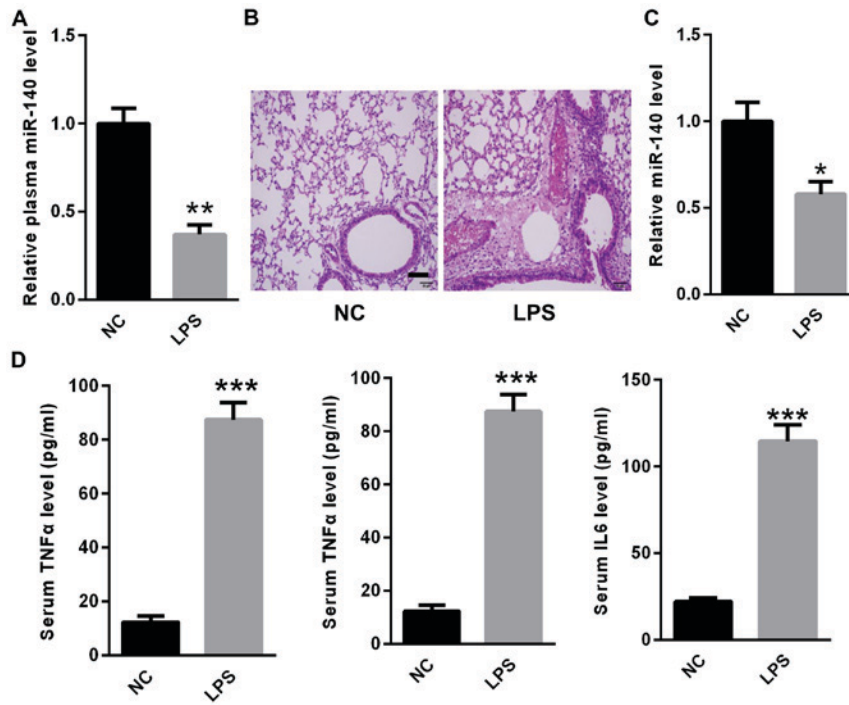
Figure 3. miR-140 expression was lower in the peripheral blood and lung tissues of rats with LPS-induced ALI. (A) miR-140 was decreased in the peripheral blood of rats in the LPS group compared with NC rats. (B) Hematoxylin and eosin staining revealed that rats in the LPS group developed obvious lung inflammation, hemorrhaging and alveolar septal thickening compared with the NC group. Scale bar=25 μm (C) miR-140 expression was decreased in the lung tissues of LPS rats compared with NC rats. (D) ELISA results revealed that serum levels of TNF-α, IL-6 and IL-1β were significantly increased in ALI rats compared with NC rats. *P<0.05, **P<0.01 and ***P<0.001 vs. NC. miR, microRNA; LPS, lipopolysaccharide; ALI, acute lung injury; NC, negative control; TNF, tumor necrosis factor; IL, interleukin.
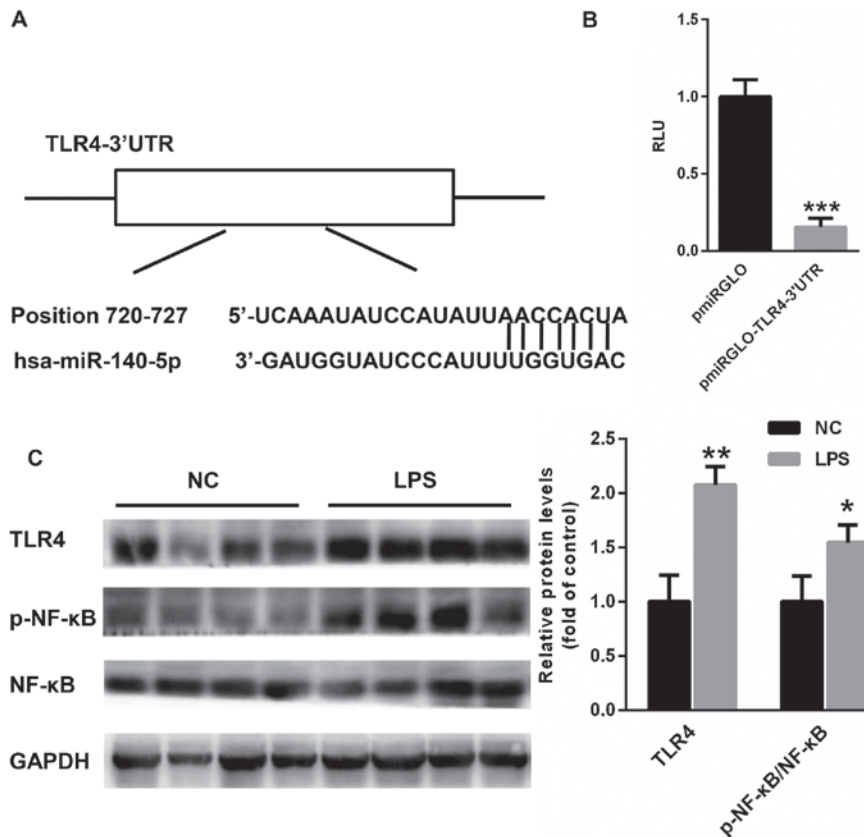

Figure 4. TLR4 was a target gene of miR-140. (A) A conserved binding site of miR-140 was identified on the 3'UTR of TLR4. (B) Dual luciferase reporter assay results suggested that miR-140 significantly suppressed the relative luciferase activity of pmirGLO-TLR4-3'UTR. (C) Western blotting revealed that expression of TLR4 was increased in the lung tissues of LPS rats compared with NC rats. *P<0.05, **P<0.01 and ***P<0.001 vs. control. TLR4, toll-like receptor 4; miR, microRNA; UTR, untranslated region; LPS, lipopolysaccharide; NC, negative control; p, phosphorylated; NF, nuclear factor.
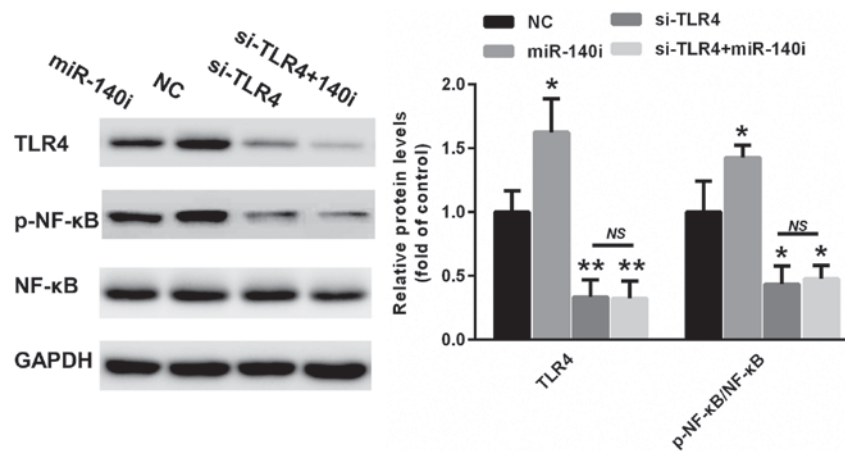
Figure 5. TLR4 silencing suppresses the phosphorylation of NF-κB even in A549 cells transfected with miR-140 inhibitor. *P<0.05 and **P<0.01 vs. NC, ns represents none sense vs. as indicated. TLR4, toll-like receptor 4; NF, nuclear factor; miR, microRNA; si, small interfering RNA; p, phosphorylated; NC, negative control.

decade, some studies have focused on the potential application of circulating miRNAs as novel prognostic and therapeutic biomarkers (33,34). miRNAs target multiple genes and may affect different signaling pathways (35,36). miRNAs may suppress inflammation pathway genes and lead to abnormal changes in the acute inflammatory response in patients under mechanical ventilation (35,36). In 2008, miRs were first identified in human blood (37), suggesting the potential application of peripheral blood or serum microRNAs as biomarkers for neoplastic and non-neoplastic diseases (37,38).

In the focus of the present study was miR-140, which is poorly understood in the progression of ALI. The results revealed that miR-140 is lower in the peripheral blood of patients with ALI than in healthy subjects. ROC analysis indicated that miR-140 could be used to screen ALI patients from healthy controls. Furthermore, decreased miR-140 expression was observed in the plasma and lungs of rats with ALI compared with controls. As miRNA mainly exerts its effects through target genes, bioinformatics was used to establish TLR4 as a target gene of miR-140 in the progression of ALI. TLR4 silencing was demonstrated to suppress the phosphorylation of NF-κB even in A549 cells transfected with miR-140 inhibitor. Taken together, these results indicate that miR-140 downregulation and increased TLR4 signaling activation may serve important roles in the lungs following LPS-triggered inflammation.

In conclusion, to the best of our knowledge the present study demonstrated that miR-140 was decreased in the progression of ALI for the first time, with further study revealing that TLR4 was a target gene of miR-140. However, miRNA expression is dynamic due to the changing cellular environment. Hence, it is important to further explore the underlying network by which miR-140 is associated with the development of inflammatory lung disease. Furthermore, future study is necessary to fully elucidate whether miR-140 could be used as a therapeutic target for patients with ALI.

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Not applicable.

Availability of data and materials

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

Authors' contributions

XL performed the experiments and analyzed the data. JW, HW, PG, CW, and YW performed part of the RT-qPCR experiments. ZZ designed the experiments, analyzed the data and gave final approval of the version to be published. All authors read and approved the final manuscript.

Ethics approval and consent to participate

The present study was approved by the Research Ethics Committee of Zhongnan Hospital of Wuhan University (Wuhan, China) and all the patients have provided written informed consent for this study.

Patient consent for publication

Informed consent for participation in the study or use of their tissue was obtained from all participants.

Competing interests

The authors declare that they have no competing interests.

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