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Original Article

MiR-338-3p Is a Biomarker in Neonatal Acute Respiratory Distress Syndrome (ARDS) and Has Roles in the Inflammatory Response of ARDS Cell Models

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To investigate the association between serum miR-338-3p levels and neonatal acute respiratory distress syndrome (ARDS) and its mechanism. The relative miR-338-3p expression in serum was detected by quantitative real-time RT-PCR. Interleukin-1beta (IL-1β), IL-6, and tumor necrosis factor-alpha (TNF-α) levels were detected by ELISAs. A receiver operating characteristic (ROC) curve analysis of serum miR-338-3p evaluated the diagnosis of miR-338-3p in neonatal ARDS. Pearson's correlation analysis evaluated the correlation between serum miR-338-3p and neonatal ARDS clinical factors. Flow cytometry evaluated apoptosis, and a CCK-8 assay assessed cell viability. A luciferase assay evaluated the miR-338-3p/AKT3 relationship. The miR-338-3p expression was decreased in neonatal ARDS patients and in lipopolysaccharide (LPS)-treated cells. The ROC curve showed the accuracy of miR-338-3p for evaluating neonatal ARDS patients. The correlation analysis demonstrated that miR-338-3p was related to PRISM-III, PaO_2/FiO_2, oxygenation index, IL-1β, IL-6, and TNF-α in neonatal ARDS patients. MiR-338-3p overexpression inhibited the secretion of inflammatory components, stifled cell apoptosis, and LPS-induced advanced cell viability. The double-luciferase reporter gene experiment confirmed that miR-338-3p negatively regulates AKT3 mRNA expression. Serum miR-338-3p levels were related to the diagnosis and severity of neonatal ARDS, which may be attributed to its regulatory effect on inflammatory response in ARDS.

Key words: miR-338-3p, AKT3, neonatal ARDS, inflammation, diagnosis

Acute respiratory distress syndrome (ARDS) is an acute pulmonary inflammatory disease in newborns that is caused mainly by a variable secondary impairment of surfactant function or the surfactant amount and extensive lung tissue inflammation [1, 2]. The Montreux definition of neonatal ARDS (2017) defines ARDS patients based on five features: the timeframe, exclusion criteria, lung imaging, the origin of edema, and oxygenation deficit [2]. The defined criteria of neonatal ARDS include the following: acute onset, oxygenation impairment with reduced end expiratory lung volume requiring positive pressure to recruit the alveoli, respiratory failure not fully explained by lung edema due to heart failure, and diffuse bilateral opacities with loss of aeration on chest radiographs [2]. The pathophysiology of ARDS differs from that of neonatal respiratory distress syndrome (RDS), another common disease that is defined as insufficient surfactant production or pulmonary structural immaturity and is specifically relevant to preterm newborns [1, 3, 4].

The clinical symptoms of ARDS are characterized by

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respiratory distress and progressive exacerbation shortly after birth [5]. Neonatal ARDS is a common critical disease in the neonatal period, and it has unique triggering factors such as necrotizing enterocolitis, neonatal asphyxia, choking milk, meconium aspiration syndrome, and others [6,7]. With the increase in the proportion of older mothers and the improvement in the level of assisted reproductive technology, the number of premature babies (especially ultra-immature babies) is increasing, and the probability of the occurrence of ARDS has increased accordingly. The incidence of children who have had ARDS is another serious issue. It is of great importance to establish the mechanisms underlying neonatal ARDS, particularly when ARDS is a novel diagnosis for newborns [6].

MicroRNAs (miRNAs) are non-coding small molecule RNAs that participate in the transcriptional regulation of a variety of genes [8]. miRNAs can control molecular pathways of inflammation by influencing target qualities and playing a part within the movement of inflammatory reaction [9,10]. Studies of the relationship between miRNAs and neonatal ARDS are rare, and it remains unknown whether miRNAs are involved in inflammatory regulation in the development of neonatal ARDS. It would be worthwhile to determine the effects of using a specific miRNA as an intervention target for the management of neonatal ARDS. An investigation of lipopolysaccharide (LPS)-induced acute lung injury in a murine model revealed that the concentration of the microRNA-338-3p (miR-338-3p) is attenuated [11]. In another study, the expression of miR-338-3p was lessened in LPS-treated 16HBE cells, and miR-338-3p weakened the assemblage of interleukin (IL)-6 and IL-8, indicating miR-338-3p's function in inflammatory protection [12]. Those findings indicated that miR-338-3p participates in neonatal ARDS caused by inflammation.

The present study was conducted to investigate the association between serum miR-338-3p levels and ARDS and its underlying mechanism. The changes in serum miR-338-3p were assessed, and the potential actions of miR-338-3p in the progression of ARDS were examined. The inter-relationship between miR-338-3p and neonatal ARDS was investigated, and the predictive role of miR-338-3p for screening neonatal ARDS was evaluated. The potential roles of miR-338-3p in neonatal ARDS were examined by an exploration of the actions of miR-338-3p in LPS-treated A549 cells.

Materials and Methods

Cases and specimen gathering. The cases of a total of 37 newborns diagnosed with ARDS were gathered. The diagnoses of neonatal ARDS were based on the Montreux definition of 2017 [2]. The inclusion criteria for the neonatal group were as follows: complete clinical information, diagnosed as ARDS according to the Montreux definition (2017), and available serum specimens before the initiation of treatment. The exclusion criteria included dyspnea caused by a congenital malformation, transient rapid breathing at the newborn phase, genetic defects related to a surfactant, severe liver or kidney dysfunction, and incomplete clinical data. Another 32 healthy infants were randomly selected as the controls. The included healthy individuals met the following criteria: matched age and weight of the patients with neonatal ARDS, available serum specimens, and no neonatal ARDS or other acute respiratory condition. All participants were from People's Hospital of Xingtai (China). This study was performed in accord with the Declaration of Helsinki, and the experiments were approved by the Ethics Committee of People's Hospital of Xingtai. The informed consent of the infants' family members or guardians was obtained.

The PRISM III scoring system was used to evaluate the severity of ARDS within 24 h post-admission in the pediatric intensive care unit. On the day after the diagnosis of ARDS, 2 ml of fasting venous blood was collected from the infant and placed in a centrifuge tube without anticoagulant. The supernatant was centrifuged and reserved at −80°C for testing.

Determining the serum concentrations of miR-338-3p. Each of the above-mentioned serum samples was thawed and mixed with RNA extract TRIzol LS (Invitrogen, Carlsbad, CA, USA). After being centrifuged and washed with 75% ethanol (Aladdin, Shanghai, China), the total RNA was identified by a NanoDrop spectrophotometer (ThermoFisher Scientific, Waltham, MA, USA). Only RNA satisfying an optical density OD_{260}/OD_{280} of 1.8-2.0 was used to prepare cDNA, with an miRNA cDNA Synthesis Kit (TaKaRa, Tokyo).

Quantitative RNA was detected using SYBR Green Premix Ex Taq II (TaKaRa) on an ABI 7500 fluorescence quantitative real-time reverse transcription-polymerase chain reaction (qRT-PCR) instrument (Applied Biosystems, Foster City, CA, USA). The relative expression level of miR-338-3p was calculated using the

2-ΔΔCt method. U6 was used as a housekeeping gene. The primers for miR-338-3p and U6 were as follows: miR-338-3p forward 5'-TGCGGTCCAGCATCAGTG AT-3', miR-338-3p reverse 5'-CCAGTGCAGGGTCC GAGGT-3'; U6 forward 5'-GCTCGCTTCGGCAGC ACA-3', U6 reverse 5'-GAGGTATTCGCACCAGAG GA-3'.

Cell cultivation and model establishment. A549 (adenocarcinomic human alveolar basal epithelial) cells were obtained from the cell bank of the Chinese Academy of Sciences (Shanghai, China) and were cultured in Dulbecco's Modified Eagle Medium (DMEM) with 10% fetal bovine serum (FBS) (Gibco, Grand Island, NY, USA) at 37°C. The A549 cells were divided into four groups, and when approx. 80% of the cells were fused, the control cells were given DMEM, and the LPS group was given complete culture medium with LPS reagent (1 μg/ml, Sigma-Aldrich, St. Louis, MO, USA) for 24 h. The synthetic sequences of miR-338-3p mimics and miR-338-3p negative control (NC) were purchased from GenePharma Co. (Shanghai, China). In the LPS+miR-338-3p mimic and LPS+miR-338-3p NC group, sequences of the miR-338-3p mimics and miR-338-3p NC were transfected into A549 cells using Lipofectamine 2000 transfection reagent (ThermoFisher Scientific), respectively.

Examination of inflammation. The levels of IL-6, IL-1β, and tumor necrosis factor-alpha (TNF-α) were determined by enzyme-linked immunosorbent assay (ELISA) kits (Finn Biotechnology Co., Wuhan, China) according to the manufacturer's instructions.

Cell Counting Kit-8 (CCK-8) assay. A549 cells (1×10^3 cells/well) were inoculated in 96-well plates put into an incubator and cultured for 24, 48, and 72 h. At the end of each culture period, 100 μl of medium was replaced in each well. Then, 10 μl of CCK-8 (Dojindo Laboratories, Kumamoto, Japan) was added to each well and further incubated at 37°C for 4 h. The optical density value at the wavelength of 450 nm was detected by an enzyme labeling instrument (Thermo Fisher Scientific).

Detection of the apoptotic rate. For the evaluation of the rate of apoptosis, A549 cells were digested in 0.25% trypsin (Gibco, Carlsbad, CA, USA) for the preparation of single-cell suspensions. The cells were then rinsed with phosphate-buffered saline (PBS) and adjusted to 10×10^5 cells/ml. Next, 5 μl of Annexin V-FITC was added to each well for 5 min. The cells were then added with 10 μl of propidium iodide (PI) binding buffer and 400 μl of PBS. The Annexin V-FITC and PI were from an Annexin V-EGFP Apoptosis Detection Kit (KeyGEN Biotech, Nanjing, China). The detection of apoptosis was performed immediately by a FACSCalibur flow cytometer (BD Biosciences, San Jose, CA, USA).

Luciferase activity assay. The target of miR-338-3p was searched on the Targetscan (www.targetscan.org) website. Fragments of AKT3 wild-type (AKT3-WT) and mutant AKT3 (AKT3-MUT) were synthesized by Sangon Biotech (Shanghai, China). The sequences of AKT3-WT and AKT3-MUT were constructed in pGL3 luciferase reporter vector (Promega, Madison, WI, USA) separately. For the test of whether miR-338-3p was targeted to AKT3, the AKT3-WT and AKT3-MUT vectors were co-transfected into 293T cells (ATCC, Manassas, VA, USA) with miR-338-3p NC and miR-338-3p mimics respectively, using Lipofectamine 2000. The cultured supernatant was collected, and the targeted relationship was examined by a double fluorescence kit (Yeasen, Shanghai, China) after 48 h of routine culture.

Statistical analyses. The resulting data were analyzed with SPSS 23.0 and GraphPad software. The means of samples were compared by the independent sample *t*-test and an analysis of variance (ANOVA). The counting data were examined by the χ^2-test. The efficacy of miR-338-3p for diagnosing neonatal ARDS was analyzed by obtaining the receiver operating characteristic (ROC) curve. A Pearson correlation analysis was performed to analyze the interaction. Probability (p) values < 0.05 were considered significant.

Results

Comparison of basic clinical data. The neonatal ARDS group (n = 37) was comprised of 16 males and 21 females with the mean age of 17.32 ± 5.29 days and the average body weight 2.06 ± 0.39 kg. There was no significant difference in sex, age, or body weight between the neonatal ARDS and healthy groups ($p > 0.05$, Table 1). The mean Pediatric Risk of Mortality (PRISM)-III index of the neonatal ARDS group was 13.59 ± 3.24, the oxygenation index (OI) was 9.44 ± 3.37, and the PaO_2/FiO_2 ratio was 200.49 ± 57.48 (Table 1). The neonatal ARDS group's average level of IL-1β was 76.97 ± 9.83 pg/ml; that of IL-6 was 39.16 ± 9.43 pg/ml, and that of

TNF-α was 44.50 ± 7.05 pg/ml (Table 1). The ARDS group included cases of sepsis (n = 4), premature birth (n = 11), pneumonia (n = 9), meconium aspiration syndrome (n = 8), and other causes (n = 5) (Table 1).

Serum miR-338-3p level and diagnostic efficacy. The serum miR-338-3p expression in the neonatal ARDS group was significantly diminished compared to that in the healthy group expression ($p < 0.001$, Fig. 1A), which suggests that the progression of neonatal ARDS might be conducive to alterations of miR-338-3p. The area under the ROC curve (AUC) of miR-338-3p was 0.886, with 91.9% sensitivity and 81.2% specificity at the cut-off value of 0.827 (Fig. 1B).

Correlation between miR-338-3p and clinical characteristics. Table 2 summarizes the correlations between the serum miR-338-3p levels and the clinical characteristics of the patients with ARDS, and Fig. 2 illustrates the corresponding results. A significant positive correlation between the serum miR-338-3p levels and PaO_2/FiO_2 scores was identified in the neonatal ARDS group ($r = 0.762$, $p < 0.001$, Fig. 2A). There were also significant negative interactions between the serum miR-338-3p levels and the PRISM-III score ($r = -0.727$, $p < 0.001$, Fig. 2B), the OI ($r = -0.839$, $p < 0.001$, Fig. 2C), and the levels of IL-1β ($r = -0.689$, $p < 0.001$, Fig. 2D), IL-6 ($r = -0.539$, $p = 0.001$, Fig. 2E), and

Table 1 Comparison of basic clinical situation

Indicators	Participants		*P*-value
	Neonatal ARDS group (n = 37)	Healthy group (n = 32)	
Age (days)	17.32 ± 5.29	15.37 ± 6.92	0.190
Gender (male/female)	16/21	17/15	0.413
Weight (kg)	2.06 ± 0.39	2.24 ± 0.53	0.121
PRISM-III	13.59 ± 3.24	–	–
OI	9.44 ± 3.37	–	–
PaO_2/FiO_2	200.49 ± 57.48	–	–
IL-1β (pg/ml)	76.97 ± 9.83	–	–
IL-6 (pg/ml)	39.16 ± 9.43	–	–
TNF-α (pg/ml)	44.50 ± 7.05	–	–
Causes			
Sepsis	4 (10.81)		
Premature birth	11 (29.73)		
Pneumonia	9 (24.32)		
Meconium aspiration syndrome	8 (21.62)		
Others	5 (13.51)		

ARDS, acute respiratory distress syndrome; PRISM- III, Pediatric Risk of Mortality III score; OI, oxygenation index; IL-1β, interleukin-1 beta; IL-6, interleukin-6; TNF-α, tumor necrosis factor-alpha.

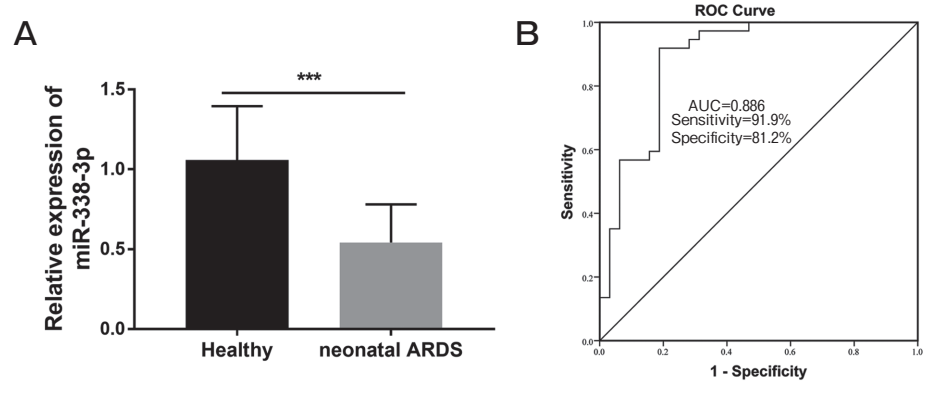

Fig. 1 The levels of miR-338-3p and its diagnostic accuracy. **A**, The reduction of miR-338-3p in the neonatal ARDS patients; **B**, The ROC curve confirmed the predictive role of miR-338-3p. ***$p < 0.001$.

Table 2 Pearson analysis on miR-338-3p and clinical data

Characteristics	Coefficient	*P*-value
PaO_2/FiO_2	0.762	<0.001
PRISM-III	−0.727	<0.001
OI	−0.839	<0.001
IL-1β (pg/ml)	−0.689	<0.001
IL-6 (pg/ml)	−0.539	0.001
TNF-α (pg/ml)	−0.604	<0.001

PRISM-III, Pediatric Risk of Mortality III score; OI, oxygenation index; IL-1β, interleukin-1 beta; IL-6, interleukin-6; TNF-α, tumor necrosis factor-alpha.

TNF-α ($r = -0.604$, $p < 0.001$, Fig. 2F) in the ARDS group.

Comparison of impacts on A549 cells among the four treatment groups. The level of miR-338-3p of the LPS group was down-regulated compared to that of the control group, but the miR-338-3p expression was significantly enhanced in the LPS+miR-338-3p mimic group ($p < 0.001$, Fig. 3A).

The effects of miR-338-3p on the activities of A549 cells induced by LPS were also evaluated. The concentrations of IL-6, TNF-α, and IL-1β in the LPS-treated cells were all greater than those of the control group, whereas the IL-6, TNF-α, and IL-1β levels in the

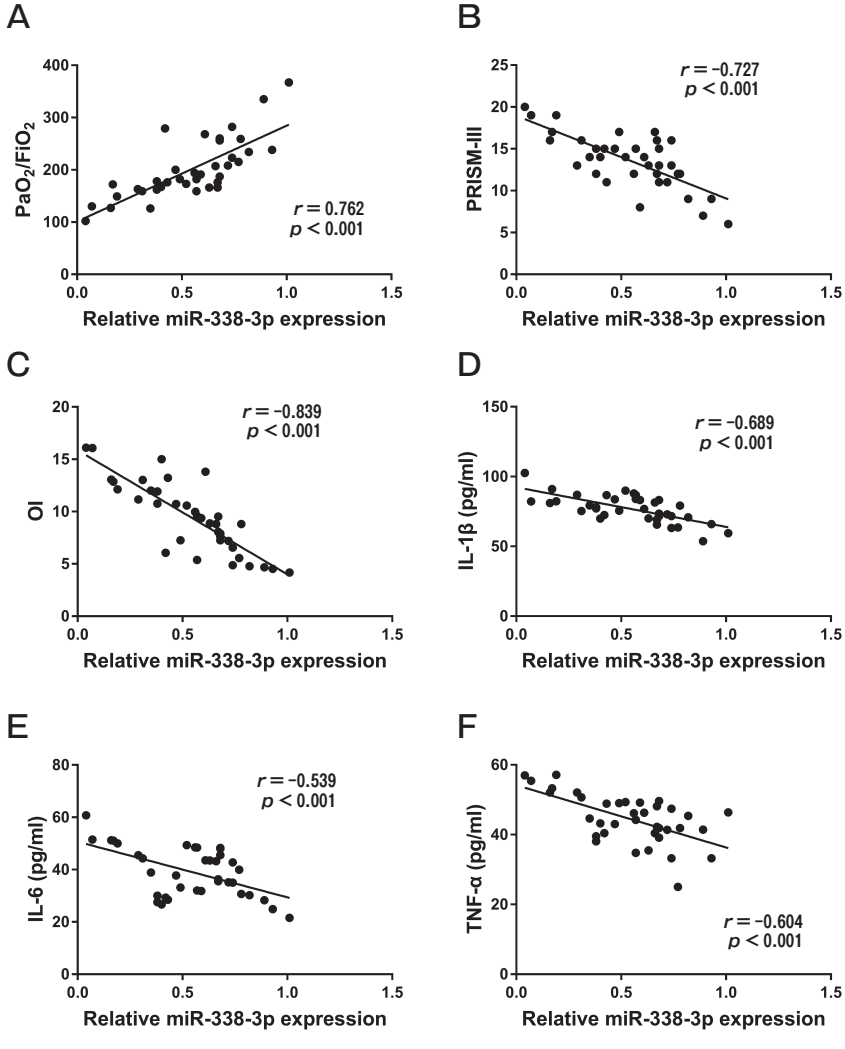

Fig. 2 The correlation between the relative miR-338-3p expression and clinical parameters in the neonatal ARDS group. **A**, The relationship between the PaO_2/FiO_2 ratio and the expression of miR-338-3p; **B**, The relative expression of miR-338-3p was correlated with the PRISM-III score; **C**, The levels of miR-338-3p were associated with the oxygenation index (OI) in the neonatal ARDS patients; **D-F**, The expression of miR-338-3p was linked to the levels of inflammatory cytokines.

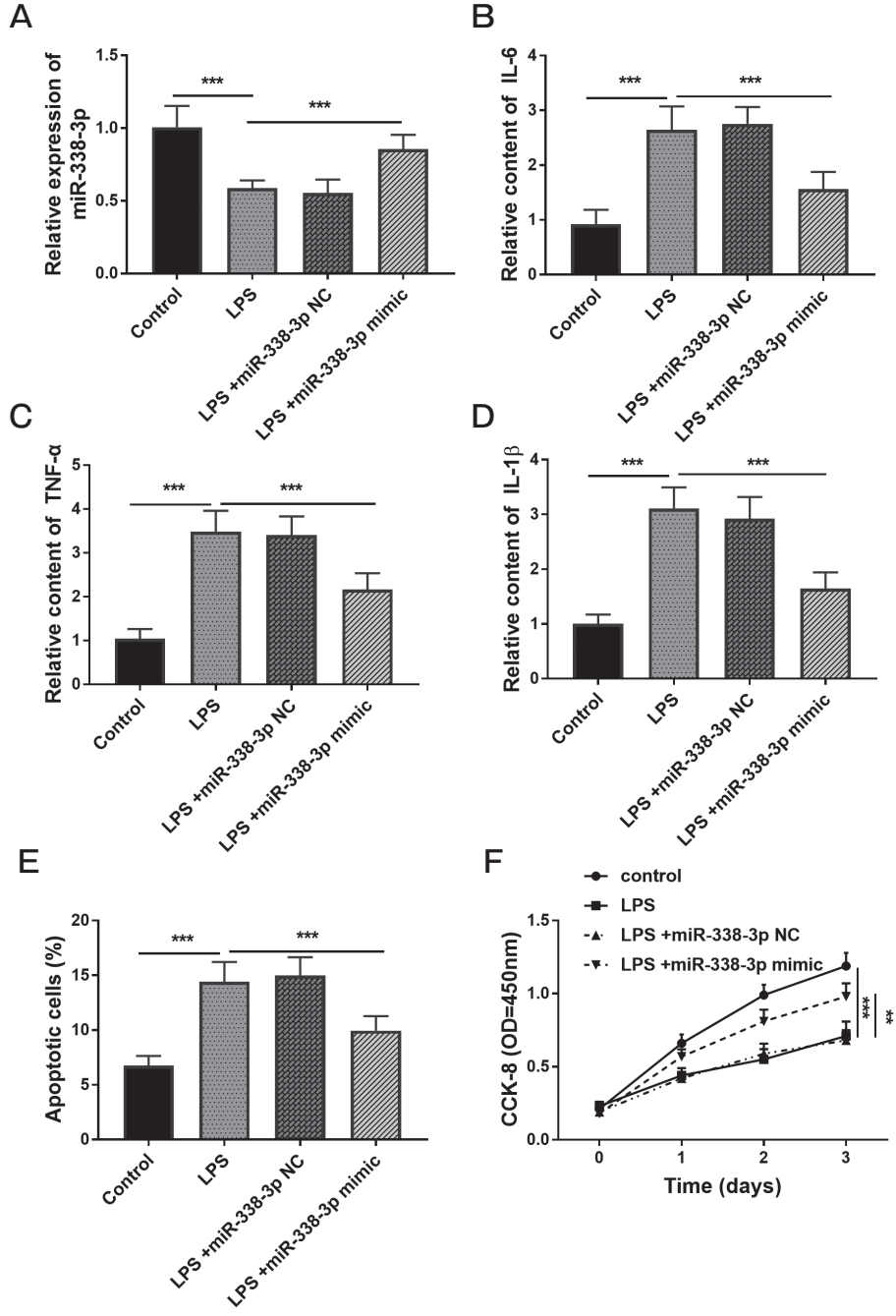

Fig. 3 The effects of miR-338-3p on LPS-treated A549 cells. **A**, LPS contributed to the diminished expression of miR-338-3p, and the miR-338-3p mimic reversed this tendency; **B-D**, The upregulation of miR-338-3p was beneficial to the recovery of cell inflammation; **E**, MiR-338-3p reversed the abnormal apoptosis of A549 cells induced by LPS; **F**, Overexpression of miR-338-3p protected the viability of A549 cells from LPS injury. $^{**}p<0.01$, $^{***}p<0.001$.

LPS+miR-338-3p mimic group were significantly reduced ($p<0.001$, Fig. 3B-D). The treatment with LPS also contributed to the enhanced apoptotic rate of A549 cells compared to the control cells, but the upregulation of miR-338-3p reversed the enhancement of the apoptotic rate ($p<0.001$, Fig. 3E). The cell survival level

in the LPS group was significantly reduced, whereas the cell survival rate of the LPS+miR-338-3p mimic group was partially restored ($p < 0.01$, Fig. 3F).

AKT3 serves as a target of miR-338-3p. The use of the bioinformatics software revealed that miR-338-3p could bind to the 3' UTR (untranslated region) of AKT3 (Fig. 4A). The results of the double-luciferase reporter gene experiment confirmed that the enhancement of miR-338-3p expression inhibited the transcriptional activity of AKT3, but the inhibition disappeared after mutation in the 3' UTR region of AKT3 ($p < 0.01$, Fig. 4B). In addition, the mRNA expression of AKT3 was increased in the LPS-treated group and reversed by the augmentation of miR-338-3p ($p < 0.01$, Fig. 4C).

Discussion

ARDS is a process of diffuse lung injury, and its pathological features on imaging are irregular alveolar exudation in multiple lung fields of bilateral lung tissue [13]. An uncontrolled inflammatory response is a key link in ARDS [14]. Acute uncontrolled inflammatory reactions caused by various pathogenic factors in and outside of the lungs can affect lung tissue and cause pulmonary edema, leading to increased permeability of the pulmonary capillary endothelial cell barrier (and of A549 cells) [15]. Edema fluids rich in proteins and inflammatory cells enter the alveolar cavity and pulmonary interstitium, resulting in ARDS [16].

Excessive inflammatory reactions can also lead to diffuse alveolar-capillary membrane injury, leading to the further development of ARDS [17]. Newborns show differences in lung biology, maturity, susceptibility to bronchopulmonary dysplasia, and immune dysfunction. The clinical consequences of neonatal ARDS are thus often more serious than those of adult ARDS. At present, the diagnostic standards fir ARDS in adults are widely recognized, but there is no consensus regarding the diagnostic standards of neonatal ARDS. There is also a lack of unified prevention and treatment measures for neonatal ARDS, which results in high mortality. It is thus very important to evaluate the severity and prognosis of early neonatal ARDS in order to provide treatment and improve the prognosis.

As a type of gene regulatory molecule, miRNAs can regulate inflammatory pathways and immune response in ARDS [18]. For example, the miR-127 expression in ARDS patients is increased and is related to the severity of ARDS [19]. It was also reported that miR-574-5p suppressed the inflammatory response of ARDS via

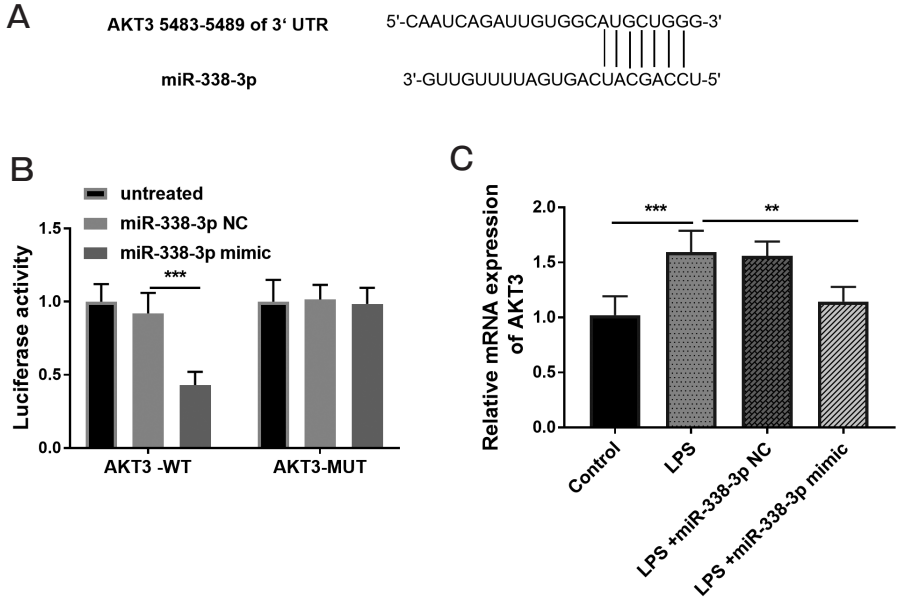

Fig. 4 The target gene of miR-338-3p. A, miR-338-3p was complementary to the 3' UTR of AKT3; B, Luciferase activity was evaluated to investigate the complementary relationship between miR-338-3p and AKT3; C, The effects of LPS and miR-338-3p expression on AKT3 mRNA expression. $^{**}p < 0.01$, $^{***}p < 0.001$.

high mobility group box 1 protein (HMGB1) [20]. In the present study, the relative expression of serum miR-338-3p in the ARDS group was decreased, indicating that miR-338-3p might be associated with the occurrence of neonatal ARDS. The relative levels of serum miR-338-3p in the present neonatal ARDS newborns were correlated with the PRISM-III, OI, and PaO_2/FiO_2 values, suggesting that miR-338-3p might be pertinent to the severity of neonatal ARDS. Moreover, miR-338-3p showed high diagnostic accuracy for differentiating neonatal ARDS patients from healthy newborns.

IL-1β, IL-6, and TNF-α are crucial inflammatory stress factors of ARDS [21]. The findings obtained herein confirmed links between miR-338-3p and these inflammatory activators, suggesting that miR-338-3p might participate in the progression of neonatal ARDS through the inflammatory response.

The present *in vitro* experiment demonstrated that in A549 cells, (*i*) treatment with LPS impeded miR-338-3p expression and increased the levels of inflammatory factors, and (*ii*) the secretion of inflammatory factors triggered by LPS was significantly decreased under high miR-338-3p expression, indicating that miR-338-3p could effectively inhibit the inflammatory response of A549 cells induced by LPS. The beneficial regulation of miR-338-3p has been observed in several disorders, such as acute ischemic stroke and pneumonia [12, 22]. MiR-338-3p exerted a protective effect in acute liver inflammation induced by acetaminophen [23]. Other studies documented that apoptosis occurs in different periods of acute lung injury and that excessive apoptosis aggravates the pathological changes of acute lung injury [24, 25]. In the present investigation, the apoptosis rate stimulated by LPS was increased, and the apoptosis induced by LPS was effectively inhibited by the overexpression of miR-338-3p. These results suggest that miR-338-3p could alleviate the injury of alveolar epithelial cells induced by LPS. The effect of miR-338-3p on cell viability also substantiated the possible protective role of miR-338-3p in neonatal ARDS.

MiRNAs play a regulatory role by completely or incompletely pairing with the 3' UTR of a target gene, degrading the target mRNA or inhibiting its translation [26]. It is therefore necessary to identify the target gene of an miRNA in order to determine its regulatory effects. A recent study demonstrated that AKT3 plays a role in tumor regulation [27]. Bioinformatics predictions revealed the presence of an miR-338-3p complementary site at the 3' UTR position of AKT3, which might be a potential downstream target gene of miR-338-3p; the present study thus focused on the targeted regulation of AKT3 of miR-338-3p. The results of the double-luciferase reporter gene experiment showed that the transfection of miR-338-3p mimics can reduce the relative luciferase value of the AKT3-WT plasmid. The qRT-PCR results showed that the miR-338-3p mimic reversed the elevated mRNA level of AKT3 induced by LPS. Several other investigations also suggest that the miR-338-3p is a signaling component of AKT3 [28, 29]. However, there are several limitations of the present study to address, including its small sample size and the single source. Further large-scale multicenter and prospective studies are necessary to validate the clinical application significance of miR-338-3p in neonatal ARDS and its relationship with disease severity and inflammatory factors.

In summary, the results of this study are as follows. MiR-338-3p may affect the inflammatory response, apoptosis, and survival rate of A549 cells induced by LPS. AKT3 was a target of miR-338-3p. The relative expression of miR-338-3p was decreased in patients with neonatal ARDS, and the expression of miR-338-3p was related to the inflammatory reactions and severity of neonatal ARDS. MiR-338-3p might be a useful biological index to predict the occurrence of neonatal ARDS.

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