The Functional Mechanisms of miR-30b-5p in Acute Lung Injury in Children

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Background: Acute lung injury in children is a complicated disease linked to the inflammation response. MicroRNA (miRNA) plays a vital role in acute lung injury. However, the role of miR-30b-5p in the pathogenesis of acute lung injury is not clear. The purpose of our study was to investigate the alteration of miR-30b-5p, suppressor of cytokine signaling 3 (SOCS3), in children with acute lung injury, and also in a mouse model of acute lung injury induced by the endotoxin lipopolysaccharide (LPS).

Material/Methods: The levels of miR-30b-5p, SOCS3, FKN (fractalkine), tumor necrosis factor (TNF)-α, NF-κB (nuclear factor kappa-light-chain-enhancer of activated B), interleukin-6 (IL-6), and IL-8 were detected by ELISA (enzyme-linked immunosorbent assay), western blot, and qRT-PCR (quantitative reverse transcription polymerase chain reaction) assay. The alveolar permeability index and the ratio of wet weight/dry weight (W/D) were measured. Then, we examined the inflammation and apoptosis using hematoxylin and eosin (H&E) staining and TUNEL (terminal deoxynucleotidyl transferase dUTP nick end labeling) assay. Additionally, SOCS3 was investigated as a direct target of miR-30a-5p in RAW264.7 cells by dual-luciferase reporter assays.

Results: Our study indicated that the level of miR-30b-5p was decreased and the levels of SOCS3, FKN, TNF-α, NF-κB, IL-6, and IL-8 were increased in lung tissue, serum, and bronchoalveolar lavage fluid of mice with acute lung injury induced by LPS. In addition, LPS increased alveolar permeability index and the ratio of W/D and induced inflammatory responses, including the activation of the NF-κB pathway in a mouse model. Furthermore, SOCS3 was confirmed to be a target of miR-30a-5p in RAW264.7 cells.

Conclusions: Our data demonstrated an important role for miR-30b-5p in acute lung injury inflammation and suggested that miR-30b-5p might be an important therapy target in children with acute lung injury.

MeSH Keywords: Acute Lung Injury • Lipopolysaccharides • MicroRNAs • Suppressor of Cytokine Signaling Proteins

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**Background**

Acute lung injury (ALI) involves pulmonary inflammation that can result in pneumonia, pulmonary edema, aspiration syndromes, hypoxemia, and respiratory failure [1,2]. The overall incidence of pneumonia in children is high worldwide [3]. Previous studies have shown that ALI is associated with pediatric intensive care unit admissions [4,5]. Therefore, there is increasing interest in developing effective biomarkers ALI that would be helpful for early diagnosis in children. Lipopolysaccharide (LPS) induced ALI is commonly used as the animal model in studies. LPS-treated mice can result in lung inflammation resolution and fibrosis [6]. Exploring effective methods for managing ALI, and finding the molecular mechanism are very important in the study of ALI.

MicroRNAs (miRNAs) are a type of small (19–24 nucleotides in length), non-coding RNAs molecules that can translational-ly inhibit gene expression or directly target mRNA for degradation by binding to 3’ untranslated region (UTR) [7,8]. MiRNAs play an important role in regulation of cell proliferation, differentiation, apoptosis, metabolism, and immunity. MiR-155 is a pro-inflammatory factor as the miR-155 gene inactivation has been shown to protect mice from LPS-induced ALI [9]. In studies of pro-inflammatory cytokines, miR-155 expression was downregulated and anti-inflammatory cytokines tumor necrosis factor (TNF-α), interleukin-12 (IL-12), and IL-10 were notably increased [10]. Studies have shown that miR-34b-5p inhibition attenuates lung inflammation and apoptosis in LPS-induced ALI by targeting progranulin [11]. In addition, miR-300 serves as a potential biomarker of LPS-induced ALI by targeting IkBα [12]. It was previously reported that miR-30b-5p functions as a tumor suppressor in renal cell carcinoma cell proliferation and epithelial-to-mesenchymal transition by targeting G-protein subunit α-13 [13]. MiR-455-5p promotes cell proliferation and invasion by targeting suppressor of cytokine signaling 3 (SOCS3) in non-small cell lung cancer [14]. However, the expression and regulatory mechanism of miR-30b-5p needs to be further investigated in ALI.

SOCS3 belongs to a family of 8 proteins (SOCS1 to SOCS7 and CIS) that regulate the production of several cytokines [15]. It was recently reported that SOCS3 expression was upregulated in ALI [16,17]. SOCS3 participates in the regulation of lung cell inflammation though negatively regulating the JAK/STAT3 signaling pathway in LPS-induced ALI inflammation *in vivo* [18]. The role of SOCS3 in ALI has been studied in animal models. The lack of SOCS3 increased LPS-induced ALI through modulation of Ly6C (+) macrophages [19]. Thus, SOCS3 has an immune regulatory role in the pathogenesis of ALI. However, it is not well understood how SOCS3 regulates lung inflammation in ALI.

Inflammation and apoptosis play vital roles in the development of ALI. Previous studies have shown that inflammatory cytokine levels affect the severity of ALI [20,21]. Inflammation is well known to be involved in the development of ALI and accompanied with pro-inflammatory cytokine release, including TNF-α, NF-κB (nuclear factor kappa-light-chain-enhancer of activated B), IL-6, and IL-8.

In the present study, we examined how the alteration of miR-30b-5p expression was downregulated and SOCS3, fractalkine (FKN), TNF-α, NF-κB, IL-6, and IL-8 expressions were upregulated in children with ALI. Next, we detected how the expression of miR-30b-5p was decreased and the expression of SOCS3, FKN, TNF-α, NF-κB, IL-6, and IL-8 were increased in lung tissue, serum, and bronchoalveolar lavage fluid (BALF) of mice with ALI induced by LPS. We explored the correlation between miR-30b-5p and inflammation in children with pneumonia to develop a new therapeutic approaches for children pneumonia.

**Material and Methods**

**Patients and tissue samples**

From January 2016 to November 2017, 30 patients with infantile pneumonia (17 boys and 13 girls; age range 1 to 12 years, average 6.72±1.14 years) were selected as the experimental group and 30 healthy children (16 boys and 14 girls; ages range 0.8 to 12 years, average 6.87±1.43 years) were selected as the control group. Thirty patients were enrolled according to the diagnostic criteria of acute pediatric pneumonia, and the healthy children in the control group had normal physical examination results. Patients were excluded if they had endocrine system disease, nervous system disease, disease of the blood system, or failure of liver, heart, kidney or other organs. General data of the enrolled patients in the 2 groups were comparable. Fasting peripheral venous blood (3 mL) was collected from the patients and the normal healthy children. After centrifugation at 2000 rev/min, the blood samples were immediately snap-frozen in liquid nitrogen at −80°C. The ethics committee of the Puali Hospital of Wuhan approved this study, and written informed consent was obtained for all the children.

**Animal treatment**

Specific-pathogen-free BALB/c mice (21 day-old and weight range: 45 g to 60 g) were purchased from the experimental animal center. The animals were divided into the two groups and treated as indicated: normal control group (0.9% sodium chloride) and ALI group (treated with 4 mg/kg LPS). Blood (0.5 mL/each mouse) and BALF (2 mL) was collected and centrifuged for following study. Whole lung tissue was carefully harvested at 4°C, and then immediately frozen in liquid nitrogen and stored at −80°C.
Measurements of alveolar permeability index

The blood was collected from inferior vena cava, then upper layer of serum was collected after centrifugation for 10 minutes. The total amount of serum protein was determined by BCA method. The chest was opened, and the trachea was cut, then a 12-gauge needle was inserted to inject 2 mL free water from the air tube and prevent BALF spillover by ligation of the suture. BALF (2 mL) was collected and centrifuged for 10 minutes at 2500 rpm/min. Supernatant was collected and stored at −80°C for the measurement of protein. The total amount of BALF protein was determined by the BCA method. Measurements of alveolar permeability index was serum protein/BALF protein.

Measurements of the ratio of wet weight/dry weight (W/D)

To quantify the extent of pulmonary edema, we evaluated the wet weight/dry weight (W/D) ratio of the lung. After rats were sacrificed, the lungs were isolated after chest opening, and the right superior lobe was cut out. The measurements of the wet weight (W) were detected from right lungs. The right lung was dried at 80°C for 24 hours, then dry weight (D) was detected. The W/D was calculated to assess tissue edema. Lung W/D ratio was calculated as described previously [22,23].

Hematoxylin and eosin (H&E)

Lungs were fixed with 10% buffered formalin at room temperature for 48 hours, dehydrated, and embedded in paraffin. The sections were stained with hematoxylin and eosin (H&E). A board-certified pathologist examined lung pathological alterations.

Apoptosis analysis

Apoptosis was analyzed using a terminal deoxynucleotidyltransferase-mediated dUTP-biotin nick end labeling (TUNEL) assay following the manufacturer’s instructions. In brief, cells were fixed, permeabilized, and incubated with fluorescein isothiocyanate (FITC)-labeled dUTP and terminal deoxynucleotidyltransferase for 1 hour at 37°C. Nuclei counterstaining was performed using DAPI. Stained cells were examined under a microscope (Leica Microsystems Wetzlar GmbH, Germany). A board-certified pathologist examined lung pathological alterations.

Enzyme-linked immunosorbent (ELISA) assay

Blood serum and BALF were analyzed using SOCS3, FKN, IL-6, and IL-8 ELISA kits (R&D Systems) according to the manufacturer instructions. Concentration of SOCS3, FKN, IL-6, and IL-8 were determined as pg/mL based on the appropriate standard curve.

Immunohistochemistry analysis

For immunohistochemistry analysis, the fresh lung tissue samples were fixed in formalin for 48 hours. Then the tissue block was put into paraffin and then cut into slides for the desired thickness using a microtome; then the slides were fixed. After washing, the samples were prepared for blocking and incubating with antibody SOCS3, FKN, TNF-α, and NF-κB, which were diluted in 5% horse serum with chilled phosphate buffered saline at 4°C overnight. Isotype-matched IgG was used instead of primary antibody as the negative control for the staining. Sections were then incubated with diluted streptavidin-peroxidase horseradish peroxidase at room temperature with a staining kit, following the manufacturer’s instructions. The sections were then stained with hematoxylin for 5 minutes, mounted, and observed with a phase-contrast microscope.

Western blot assay

Lung tissue, blood serum, and BALF were separated by 10% SDS-PAGE. Separated proteins were electrophoretically transferred to polyvinylidene fluoride (PVDF) membrane and blocked for 2 hours at room temperature with TBS containing 5% BSA. The primary antibodies were as follows: SOCS3 (1: 500), FKN (1: 1000), TNF-α (1: 500), NF-κB (1: 1000), IL-6 (1: 1000), and IL-8 (1: 1000) at 4°C overnight. After incubating with horseradish peroxidase-conjugated goat anti rabbit IgG, protein bands were detected using an increased chemiluminescence detection kit.

Cell culture and treatments

The mouse macrophage cell line RAW264.7 was cultured in DMEM medium in a humid atmosphere with 5% CO₂ and 95% air; then cells were cultured with 10 ng/mL LPS in media under normoxia conditions until 70% to 80% confluence. After RAW264.7 cells were stimulated with LPS for 48 hours, cells were transfected with miR-30b-5p mimics or control mimics. The groups were designed as follows: negative control (NC) group, miR-30b-5p mimics group, and control mimics group. Subsequently, the transduction efficiency was verified by quantitative reverse transcription polymerase chain reaction (qRT-PCR). The mRNA level of SOCS3 was measured simultaneously. After that, RAW264.7 cells were seeded into 96-well plates, and a pGL3 firefly luciferase reporter gene vector (Promega Corporation, Madison, WI, USA) with the 3′-UTR-wildtype (WT) or 3′-UTR-mutant (MUT) fragment of SOCS3 cDNA, containing a putative target site for miR-30b-5p, was co-transfected with miR-30b-5p mimics and control mimics into RAW264.7 cells. At 48 hours after transfection, the relative luciferase activity of the cell extracts was measured using the Dual-Luciferase reporter system.

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to the control group (\(^*\) \(P<0.05\)). In addition, the ratio of W/D significantly increased in mice with ALI compared to the control group (Figure 2B, ** \(P<0.01\)).

**Effects of inflammation and cell apoptosis in mice with ALI**

LPS induction is well known to cause injury in different organs, including heart, liver, renal, and even the lung, and has been investigated previously [24]. Inflammation and apoptosis play vital roles in the development of ALI. We next focused our study on miR-30b-5p regulation on inflammation and cell apoptosis in mice with ALI. In this study, H&E staining was used to assess inflammatory cell infiltration in the lungs of the mice. The lung tissue in the ALI group showed the following signs: destroyed, edematous, small alveoli with hemorrhagic or dilated capillaries, pneumatocele, and thickened alveolar septum (Figure 2C). In addition, the results of TUNEL indicated that the percentage of TUNEL-positive cells in the ALI group was markedly increased compared with the control group (Figure 2D). Thus, these results suggested that the ALI models were successfully established, and induced inflammation and cell apoptosis in mice with ALI.

**Expression of SOCS3, FKN, TNF-\(\alpha\), NF-\(\kappa\)B, IL-6, and IL-8 attribute to inflammatory response in lung tissue, serum, and BALF of mice with ALI**

Inflammation response is known as a major reason to cause tissue injury in liver and renal organs injury after LPS treatment [25]. Previously studies reported that LPS treatment resulted in lung injury which was attributed to inflammatory response [26]. NF-\(\kappa\)B signaling pathway is a key to modulate pro-inflammatory cytokine releases [27]. We assessed the expression of SOCS3, FKN, TNF-\(\alpha\), and NF-\(\kappa\)B in lung tissue, serum and BALF of mice with ALI. As shown in Figure 2E, TNF-\(\alpha\) was located in the cell membrane or cytoplasm, both of which contained brownish yellow particles. The positive rate of TNF-\(\alpha\) protein in ALI lung tissue was remarkably higher than that in normal lung tissue. We found that pro-inflammatory cytokines of SOCS3, FKN, TNF-\(\alpha\), and NF-\(\kappa\)B were marked upregulated in lung tissue of mice with ALI compared with the control group. As shown in Figure 3, we found that pro-inflammatory cytokines of IL-6, IL-8, SOCS3, and FKN were significantly upregulated in serum and BALF of mice with ALI (** \(P<0.01\), *** \(P<0.001\)). These findings suggest that LPS induced an inflammation response and that SOCS3 plays a certain role in this process.

**The mRNA levels of miR-30b-5p, SOCS3, FKN, TNF-\(\alpha\), NF-\(\kappa\)B, IL-6, and IL-8 in mice with ALI**

As mentioned, the inflammation response has been observed in LPS-induced mice with ALI. As shown in Figure 4A, the mRNA
Figure 1. The levels of miR-30b-5p, SOCS3, FKN, TNF-α, NF-κB, IL-6, and IL-8 in children with pneumonia. (A) miR-30b-5p expression was downregulated in the experimental group compared with the control group. (B-G) The mRNA levels of SOCS3, FKN, TNF-α, NF-κB, IL-6, and IL-8 were upregulated in patients in the experimental group comparing to the control group. (** P<0.01, *** P<0.001, experiment versus control.) SOCS3 – suppressor of cytokine signaling 3; FKN – fractalkine; TNF-α – tumor necrosis factor-α; NF-κB – nuclear factor kappa-light-chain-enhancer of activated B; IL-6 – interleukin-6; IL-8 – interleukin-8.
expression of miR-30b-5p was decreased significantly (** P<0.01). However, the mRNA levels of SOCS3, FKN, TNF-α, NF-κB, IL-6, and IL-8 were increased markedly in lung tissue of mice with ALI compared with the control group. (A) The alveolar permeability index was significantly increased in mice with ALI compared to the control group. (B) The ratio of W/D was significantly increased in ALI group compared with the control group. (* P<0.05, ** P<0.01, ALI versus control). (C) Representative images of lung injury by hematoxylin and eosin staining. (D) Representative images of apoptosis by TUNEL. (E) The expression of SOCS3, FKN, TNF-α, and NF-κB were remarkably upregulated in lung tissue of mice with ALI by immunocytochemistry analysis compared with the control group. As the picture shows, the number of brownish yellow particles in the ALI group was significantly higher than that of the control group. ALI – acute lung injury; W/D – wet weight/dry weight; TUNEL – terminal deoxynucleotidyl transferase dUTP nick end labeling; SOCS3 – suppressor of cytokine signaling 3; FKN – fractalkine; TNF-α – tumor necrosis factor-α; NF-κB – nuclear factor kappa-light-chain-enhancer of activated B.

Figure 2. The alveolar permeability index, the ratio of W/D, lung tissue injury, apoptosis, and the levels of inflammatory factors in mice with ALI. (A) The alveolar permeability index was significantly increased in mice with ALI compared to the control group. (B) The ratio of W/D was significantly increased in ALI group compared with the control group. (* P<0.05, ** P<0.01, ALI versus control). (C) Representative images of lung injury by hematoxylin and eosin staining. (D) Representative images of apoptosis by TUNEL. (E) The expression of SOCS3, FKN, TNF-α, and NF-κB were remarkably upregulated in lung tissue of mice with ALI by immunocytochemistry analysis compared with the control group. As the picture shows, the number of brownish yellow particles in the ALI group was significantly higher than that of the control group. ALI – acute lung injury; W/D – wet weight/dry weight; TUNEL – terminal deoxynucleotidyl transferase dUTP nick end labeling; SOCS3 – suppressor of cytokine signaling 3; FKN – fractalkine; TNF-α – tumor necrosis factor-α; NF-κB – nuclear factor kappa-light-chain-enhancer of activated B.

Validation of SOCS3 as a direct target of miR-30b-5p in RAW264.7 cells

The complimentary sequences for miR-30b-5p in the 3’UTR of SOCS3 were obtained from the TargetScan database (Figure 6A).
Figure 3. Expression of SOCS3, FKN, IL-6, and IL-8 in the serum and BALF of mice with ALI. The expression of SOCS3, FKN, IL-6, and IL-8 were significantly upregulated in serum (A) and BALF (B) of mice with ALI by ELISA assay. (** P<0.01, *** P<0.001, ALI versus control.) ALI – acute lung injury; SOCS3 – suppressor of cytokine signaling 3; FKN – fractalkine; IL-6 – interleukin-6; IL-8 – interleukin-8; BALF – bronchoalveolar lavage fluid; ELISA – enzyme-linked immunosorbent assay.
Figure 4. The mRNA levels of miR-30b-5p, SOCS3, FKN, TNF-α, NF-κB, IL-6, and IL-8 in lung tissue of mice with ALI. The qRT-PCR assay suggested that the mRNA levels of miR-30b-5p was decreased (A) and the mRNA levels of SOCS3, FKN, TNF-α, NF-κB, IL-6, and IL-8 (B–G) were increased in the lung tissue of mice with ALI compared with the control group. (** \( P < 0.01 \), *** \( P < 0.001 \), ALI versus control.) ALI – acute lung injury; SOCS3 – suppressor of cytokine signaling 3; FKN – fractalkine; TNF-α – tumor necrosis factor-α; NF-κB – nuclear factor kappa-light-chain-enhancer of activated B; IL-6 – interleukin-6; IL-8 – interleukin-8; qRT-PCR – quantitative reverse transcription polymerase chain reaction.
Figure 5. The protein levels of SOCS3, FKN, TNF-α, NF-κB, IL-6, and IL-8 in lung tissue, serum, and BALF of mice with ALI. Western blotting assay also suggested that the protein levels of SOCS3, FKN, TNF-α, NF-κB, IL-6, and IL-8 were increased in lung tissue (A), serum (B), and BALF (C) of mice with ALI compared with the control group. (** \( P < 0.01 \), *** \( P < 0.001 \), ALI versus control.) ALI – acute lung injury; SOCS3 – suppressor of cytokine signaling 3; FKN – fractalkine; TNF-α – tumor necrosis factor-α; NF-κB – nuclear factor kappa-light-chain-enhancer of activated B; IL-6 – interleukin-6; IL-8 – interleukin-8; BALF – bronchoalveolar lavage fluid.
RAW264.7 cells co-transfected with miR-30a-5p and the SOCS3 3'UTR-WT exhibited lower intracellular luciferase activity than those transfected SOCS3 3'UTR-WT (* P<0.05; Figure 6A). This data indicated that SOCS3 was negatively regulated by miR-30b-5p, and was consistent with the qRT-PCR results, which indicated that the miR-30b-5p mimics could decreased the mRNA level of SOCS3 (**) P<0.01; *** P<0.001 versus miR-30b-5p mimics control and NC group.) SOCS3 – suppressor of cytokine signaling 3.

Discussion

ALI is a common and complex inflammatory lung disease. MiRNA is a novel target of non-coding RNA molecules that regulate target gene expression, and play important roles in ALI inflammation and apoptosis. A previous study had shown that miR-34b-5p inhibition attenuated lung inflammation and apoptosis in an LPS-induced ALI mouse model by targeting progranulin [11]. In this study, compared with the normal healthy children blood samples, significant downregulation of miR-30b-5p was observed in patients and in the lung tissue, serum, and BALF of mice with ALI. We showed the expression of miR-30b-5p as biomarkers of ALI, suggesting potential use as therapeutic targets in children with ALI. In order to verify this finding, we have done related experiments in mice with ALI.

LPS induction is well known to cause injury in different organs, including the heart, liver, renal, and even the lung, and has been investigated previously [24]. In this study, first, we showed that the alveolar permeability index and ratio of W/D were increased in mice with ALI induced by LPS. We used H&E staining to assess inflammatory cell infiltration in the lungs of the mice. In addition, H&E staining analysis also showed that the degree of inflammatory infiltration in the ALI group was elevated compared to the control group. Thus, the ALI models
were successfully established, and LPS was found to severely affect inflammatory response and cell apoptosis in mice.

Inflammation and apoptosis play vital roles in the development of ALI. Furthermore, increasing evidence shows that inflammatory cytokine levels affect the severity of ALI [20,21]. Inflammation is known to be involved in ALI development and accompanied with pro-inflammatory cytokine release, including TNF-α, NF-κB, IL-6, and IL-8. Inflammation response is known as a major cause of tissue injury in the liver and renal organs after LPS treatment [25]. Previous studies reported that LPS treatment resulted in lung injury, which was attributed to inflammatory response [26]. NF-κB signaling pathway is a key to modulate pro-inflammatory cytokine releases [27]. In this study, the mRNA and protein levels of FKN, TNF-α, NF-κB, IL-6, and IL-8 were attributed to the inflammatory response in lung tissue, serum, and BALF of mice with ALI. Thus, inflammation response was observed in mice with ALI. At the same time, miR-30b-5p expression was downregulated, which revealed that miR-30b-5p might be involved in the inflammatory response of ALI to some extent.

It was previously reported that SOCS3 expression was upregulated in ALI. It was reported that lack of SOCS3 increased more inflammatory response though activation of macrophages and induced TH1/TH17 cell differentiation [17,28]. SOCS3 expression accompanied the severity of inflammation and the expression of pro-inflammatory cytokines, as well as the activation status of STAT3 and p38 MAPK [18]. In this study, we found that SOCS3 expression also was upregulated in lung tissue, serum, and BALF of mice with ALI. Based on bioinformatics predictions, SOCS3 was predicted to have a strong miR-30b-5p binding site and miR-30b-5p targeted and repressed SOCS3 directly through a 3′-UTR. In the present study, the regulatory role of miR-30b-5p to SOCS3 was clarified using luciferase-reporting system in RAW264.7 cells. Our study also showed that SOCS3 mRNA expression was downregulated by miR-30b-5p overexpression in RAW264.7 cells, thus indicating that SOCS3 was directly regulated by miR-30b-5p. Therefore, we boldly suggest that SOCS3 might be a target of miR-30b-5p in the process of regulating children with ALI. Therefore, SOCS3 has the role of both immune regulatory and pro-inflammatory immune in LPS-induced ALI. However, the specific molecular mechanism of regulation for miR-30b-5p and SOCS3 needs further experimental verification. This study only illustrates the expression trends of miR-30b-5p and SOCS3 in ALI children and mice, and the correlation with inflammatory damage, but did not provide direct evidence to prove the interaction between them. This was a limitation of this study, but also suggests the direction for our next research.

Conclusions

In conclusion, we examined the alteration of miR-30b-5p, which was downregulated, and the levels of SOCS3, FKN, TNF-α, NF-κB, IL-6, IL-8, which were upregulated in patients with pneumonia and in mice with LPS-induced ALI. LPS treatment resulted in lung injury and which was attributed to secondary inflammatory response. Therefore, focusing on suppressing inflammation factors might be a novel target in the inhibition and treatment of ALI, and miR-30b-5p might be an important target in children with ALI.

Conflict of interests

None.

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