

Clinical significance of miR-181a in patients with neonatal sepsis and its regulatory role in the lipopolysaccharide-induced inflammatory response

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Received April 29, 2019; Accepted October 30, 2019

DOI: 10.3892/etm.2020.8408

Abstract. Neonatal sepsis (NS) poses a serious threat to the health of neonates worldwide. The present study aimed to investigate the diagnostic value of microRNA (miR)-181a in patients with NS and the regulatory role of miR-181a in lipopolysaccharide (LPS)-induced inflammation. A total of 102 neonates with NS and 50 neonates without sepsis were enrolled in the present study. The serum levels of miR-181a were estimated using reverse transcription-quantitative PCR. Receiver operating characteristic (ROC) analysis was performed to evaluate the diagnostic value of miR-181a for NS. The effect of miR-181a on the expression of Toll-like receptor (TLR)4 was assessed after modification of the expression of miR-181a in monocytes isolated from the blood of neonates *in vitro*. An ELISA was used to measure the concentration of inflammatory cytokines tumor necrosis factor (TNF)- α and interleukin (IL)-8 in the supernatant of monocytes. The serum levels of miR-181a were decreased in patients with NS compared with those in the controls. The area under the ROC curve of miR-181a was 0.893 with a sensitivity of 83.3% and a specificity of 84.0%. LPS stimulation in monocytes also led to a decrease in the expression of miR-181a. TLR4 was proven to be a direct target gene of miR-181a, according to the results of a luciferase reporter assay, and overexpression of miR-181a suppressed TLR4 expression in monocytes. Regarding LPS-induced inflammation, it was revealed that the upregulated levels of TNF- α and IL-8 induced by LPS were reduced by overexpression of miR-181a in monocytes. In conclusion, decreased serum levels of miR-181a may serve as a diagnostic biomarker in patients with NS and overexpression of miR-181a inhibits the LPS-induced inflammatory response

at least partially by targeting TLR4. Aberrant miR-181a may be a non-invasive biomarker for NS patients, and provide a novel insight into the pathologic mechanisms of action behind the development of NS.

Introduction

Sepsis is a serious syndrome that is induced by infections and characterized by a systemic inflammatory response (1). Neonatal sepsis (NS) is sepsis that occurs in infants within 28 days of age and is mainly caused by bacterial infection (2). Approximately 1 million infants die from NS every year worldwide, which therefore represents a global health burden (3). However, current strategies for the diagnosis of NS remain limited. Bacterial culture is considered the gold standard for the diagnosis of NS, but it takes 1 to 2 days to obtain the examination results with low sensitivity (4). In addition, several biological markers with high sensitivity have been identified, including interleukins (ILs), C-reactive protein (CRP), micro-erythrocyte sedimentation rate and procalcitonin (PCT) (5-7). However, the clinical application of these indicators is limited due to their poor specificity. Thus, novel diagnostic biomarkers with high sensitivity and specificity are urgently required for the early diagnosis of NS.

MicroRNAs (miRNAs) are small non-coding RNAs that may be easily detected from blood samples (8). It is generally accepted that miRNAs regulate gene expression by directly binding to the 3'-untranslated region (3'-UTR) of target messenger RNA (mRNA), leading to mRNA degradation or suppression of subsequent translation (9). Furthermore, pivotal roles of miRNAs have been demonstrated in a number of biological processes, including cell proliferation, differentiation, cell cycle and cell apoptosis (10). Emerging studies have reported differentially expressed miRNAs in different types of human diseases (11-13). The clinical significance of the aberrant expression of miRNAs has attracted increasing attention due to their high diagnostic and prognostic values (14,15). Downregulated expression of microRNA-181a (miR-181a) has been identified in NS patients by Chen *et al* (16). A study by He *et al* (17) demonstrated that miR-181a improved immune thrombocytopenia by regulating Toll-like receptor 4 (TLR4), a key molecule in the innate immune system and the development of NS (18). However, the clinical and biological roles of miR-181a in NS have remained to be fully elucidated.

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Key words: microRNA-181a, neonatal sepsis, diagnosis, inflammatory response, monocyte

To improve the diagnosis of NS, the present study sought to compare the serum levels of miR-181a between NS patients and healthy newborns and explore the diagnostic value of miR-181a. Additionally, the effect of miR-181a on the lipopolysaccharide (LPS)-induced inflammatory response was further analyzed in primary monocytes.

Materials and methods

Patients and blood sample collection. The experimental protocols were approved by the Ethics Committee of Yidu Central Hospital of Weifang Hospital (Weifang, China) and written informed consent was obtained from the families of the patients. Blood samples were collected from 102 patients with NS at the time of initial laboratory evaluation at the Yidu Central Hospital of Weifang (Weifang, China) between May 2014 and April 2018, and stored at -80°C for further analysis. Furthermore, 50 neonates without any symptoms and signs of sepsis, who underwent routine consultation or vaccination at an outpatient neonatal clinic and were diagnosed with respiratory infection or pneumonia were included in the present study as a control group. The diagnosis of NS was determined based on the criteria established at the 2003 Kunming Neonatal Sepsis Definitions Conference (19); it mainly relies on the clinical manifestations and the detection of blood pathogens. *Staphylococcus* and *Escherichia coli* were the most common types among all of the detected bacteria. The clinicopathological characteristics of the participants are listed in Table I.

RNA extraction and reverse transcription-quantitative PCR (RT-qPCR). The collected blood was centrifuged to isolate the serum samples. Total RNA from the serum, including miRNAs, was extracted using TRIzol reagent (Invitrogen; Thermo Fisher Scientific, Inc.) according to the manufacturer's protocol. A NanoDrop 2000 (Thermo Fisher Scientific, Inc.) was used to evaluate the purity and concentration of the RNA. Single-stranded complementary DNA was synthesized from the RNA using the PrimeScript RT reagent kit (Takara Bio, Inc.) and stored at -20°C for subsequent qPCR. The serum levels of miR-181a and mRNA of TLR4 were determined using qPCR, which was performed using a SYBR Green I Master Mix kit (Invitrogen; Thermo Fisher Scientific, Inc.) and a 7300 Real-Time PCR System (Applied Biosystems; Thermo Fisher Scientific, Inc.). The reaction conditions are as follows: miR-181a 95°C for 10 min, 40 cycles of 95°C for 15 sec, 60°C for 20 sec, 72°C for 15 sec; TLR4 95°C for 10 min, 40 cycles of 95°C for 30 sec, 58°C for 30 sec, 72°C for 20 sec. U6 and GAPDH were respectively used as the internal control gene for miR-181a and TLR4. The final expression value was calculated using the $2^{-\Delta\Delta C_t}$ method (20) and normalized to U6 or GAPDH. The sequences of the primers used in the present study were as follows: miR-181a forward, 5'-GCCGAGAACAUUCACGCUGU-3' and reverse, 5'-CTCAACTGGTGTCTGGGA-3'; TLR4 forward, 5'-CAGAGTTGCTTTCAA TGGCATC-3' and reverse, 5'-AGACTGTAATCAAGAACC TGGAGG-3'; U6 forward, 5'-CTCGCTTCGGCAGCAC-3' and reverse, 5'-AACGCTTCACGAATTTGCGT-3'. GAPDH forward, 5'-AGAAGGCTGGGGCTCATTG-3' and reverse, 5'-AGGGGCCATCCACAGTCTTC-3'.

Cell culture and stimulation conditions. Blood samples collected from the patients with NS were settled by addition of 4.5% dextran 500 (1:5; Amersham Biosciences) and the leukocytes were separated from the red blood cells. Monocytes were isolated using density gradient centrifugation with FicollPaque (Amersham Pharmacia, Biotech AB) as previously described (21), and the purity of the cells was confirmed to be $>95\%$ by flow cytometry based on detection of the specific cell markers CD14 and CD45. The extracted monocytes were cultured in RPMI-1640 medium (Gibco; Thermo Fisher Scientific, Inc.) supplemented with 10% fetal bovine serum (Gibco; Thermo Fisher Scientific, Inc.) in a humidified atmosphere with 5% CO_2 at 37°C . To explore the effects of miR-181a on LPS-induced inflammation, the monocytes were stimulated using 100 ng/ml LPS (Sigma-Aldrich; Merck KGaA) for 4 h.

Cell transfection. Monocytes were seeded into 48-well plates and transfected with miR-181a mimics, miR-181a inhibitor and miR-negative control (miR-NC) (GenePharma) using Lipofectamine 2000 (Thermo Fisher Scientific, Inc.) according to the manufacturer's protocols. The sequences of the vectors were as follows: miR-181a mimics, 5'-AACAUUCAACGCUGUCGGUGAGU-3'; miR-181a inhibitor, 5'-ACUCACCGA CAGCGUUGAAUGUU-3'; miR-NC, 5'-CAGUACUUUUGU GUAGUACAA-3'.

Luciferase reporter assay. In a bioinformatics analysis using TargetScan (http://www.targetscan.org/vert_72/), a complementary sequence of miR-181a was identified in the 3'-UTR of TLR4. To verify whether there was a direct interaction between miR-181a and TLR4, a luciferase reporter assay was performed in the present study. The wild-type (WT) or mutant-type (MT) 3'-UTR was cloned into the pGL3 basic vector (Promega Corp.) to obtain pLUC-WT-TLR4 or pLUC-MT-TLR4, respectively. miR-181a mimics, miR-181a inhibitor or miR-NC were co-transfected into the isolated monocytes with pLUC-WT-TLR4 or pLUC-MT-TLR4 using Lipofectamine 2000 (Thermo Fisher Scientific, Inc.). A Dual-Luciferase Reporter Assay System (Promega Corp.) was used to measure the luciferase activity in the different groups.

ELISA. The concentration of the inflammatory cytokines tumor necrosis factor (TNF)- α and IL-8 in the culture supernatant of the monocytes was estimated using ELISA, which was performed using a TNF- α ELISA kit (cat. no. 550610; BD Biosciences) and an IL-8 ELISA kit (cat. no. 550999; BD Biosciences) according to the manufacturer's protocol. The optical density at 450 nm was read by using a microplate reader (Bio-Rad Laboratories, Inc.).

Statistical analysis. All statistical analyses were performed by using SPSS 18.0 software (SPSS Inc.) and GraphPad Prism 5.0 software (GraphPad Software, Inc., USA). Values are expressed as the mean \pm standard deviation and compared with Student's t-test, the χ^2 test or one-way analysis of variance followed by Tukey's multiple-comparisons test. A receiver operating characteristic (ROC) curve was drawn to evaluate the diagnostic value of miR-181a regarding NS. $P < 0.05$ was considered to indicate statistical significance.

Table I. Clinicopathological characteristics of the NS patients and the controls.

Characteristic	Control (n=50)	NS (n=102)	P-value
Age (days)	11.74±4.41	11.52±4.01	0.758
Sex (male/female)	28/22	53/49	0.730
Body weight (g)	3485.18±308.79	3472.77±299.91	0.813
CRP (mg/l)	10.89±4.74	12.46±5.86	0.099
WBC (x10 ⁹ /l)	10.62±4.92	11.59±5.77	0.307
PCT (ng/ml)	1.60±0.75	4.76±2.72	0.001

NS, neonatal sepsis; CRP, C-reactive protein; WBC, white blood cells; PCT, procalcitonin.

Results

Clinicopathological characteristics of the patients with NS and the controls. A total of 102 patients with NS and 50 controls were included in the present study. The clinicopathological characteristics, including age, sex, body weight, concentration of CRP and PCT, as well as white blood cell (WBC) count, were summarized in Table I. The control group included 28 males and 22 females with the age of 11.74±4.41 days, and 53 males and 49 females were included in the NS patients with an average of 11.52±4.01 days. There were no significant differences between the NS cases and controls in terms of age, sex, body weight, CRP and WBC count (all $P>0.05$), while a higher PCT was observed in the patients with NS compared with that in the controls ($P=0.001$).

Serum miR-181a is downregulated in patients with NS. To investigate the role of miR-181a in NS, the serum levels of miR-181a in the NS patients were measured using RT-qPCR. As presented in Fig. 1, the relative serum levels of miR-181a were significantly downregulated in patients with NS compared with those in the controls ($P<0.01$).

Diagnostic value of miR-181a in patients with NS. Given the dysregulation of miR-181a in the serum of patients with NS, its clinical significance in the diagnosis of NS was assessed in the present study. A ROC curve for PCT was first constructed based on the established diagnostic value of PCT in NS and its significantly different concentration in the present NS group compared with that in the controls. As presented in Fig. 2A, the area under the curve (AUC) for PCT was 0.874 with a sensitivity of 75.5% and a specificity of 98.0% at a cutoff value of 0.980. The ROC curve for the levels of miR-181a is presented in Fig. 2B, with an AUC of 0.893, and a sensitivity and specificity of 83.3 and 84.0%, respectively, at a cutoff value of 0.625.

Expression of miR-181a in LPS-treated monocytes. To investigate the functional role of miR-181a in LPS-induced inflammation, the expression of miR-181a in monocytes treated with LPS was measured. As presented in Fig. 3, the expression of miR-181a was obviously decreased in the monocytes after LPS stimulation compared with that in the untreated cells ($P<0.01$).

miR-181a directly regulates the expression of TLR4. TLR4 is a key molecule in the innate immune system and the development

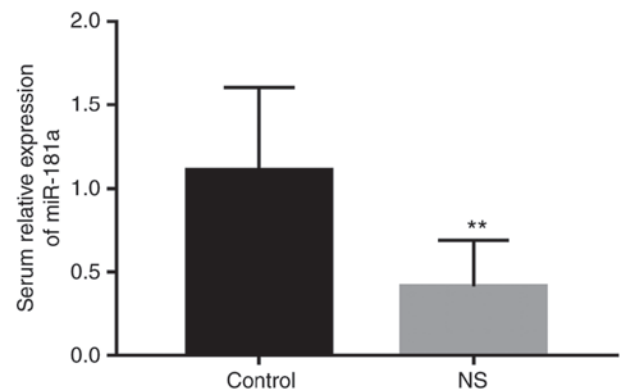


Figure 1. Serum levels of miR-181a in NS patients and controls. The expression of miR-181a was lower in the NS patients than in the controls. ** $P<0.01$ vs. Control. NS, neonatal sepsis; miR, microRNA.

of NS. TLR4 has been reported to be a direct target of miR-181a in immune thrombocytopenia (17,18). The present study focused on the association between these two molecules in the monocytes. Following transfection with miR-181a mimics or miR-181a inhibitor, the expression of miR-181a was significantly increased or decreased, respectively, as confirmed by RT-qPCR (all $P<0.01$, Fig. 4A). After the *in vitro* modification of miR-181a levels, the LPS-induced elevated expression of TLR4 was indicated to be significantly suppressed in the cells with overexpression of miR-181a, whereas it was significantly enhanced in the cells with knockdown of miR-181a (all $P<0.05$, Fig. 4B), indicating that miR-181a in LPS-treated monocytes led to inhibition of TLR4. In order to further confirm the direct interaction between miR-181a and TLR4, a luciferase reporter assay was performed. A complementary sequence of miR-181a was identified in the 3'-UTR of TLR4 (Fig. 4C). After co-transfection of the reporter vector containing the 3'-UTR sequence of TLR4 and miR-181a mimics or inhibitor, it was observed that the relative luciferase activity in the WT-TLR4 group was markedly decreased in the presence of miR-181a mimics but was increased in the presence of miR-181a inhibitor (all $P<0.05$, Fig. 4D). However, no significant changes in luciferase activity were observed in the MT-TLR4 groups.

Effects of miR-181a on the levels of pro-inflammatory cytokines in monocytes. The effects of miR-181a on inflammatory cytokines were then investigated to demonstrate the

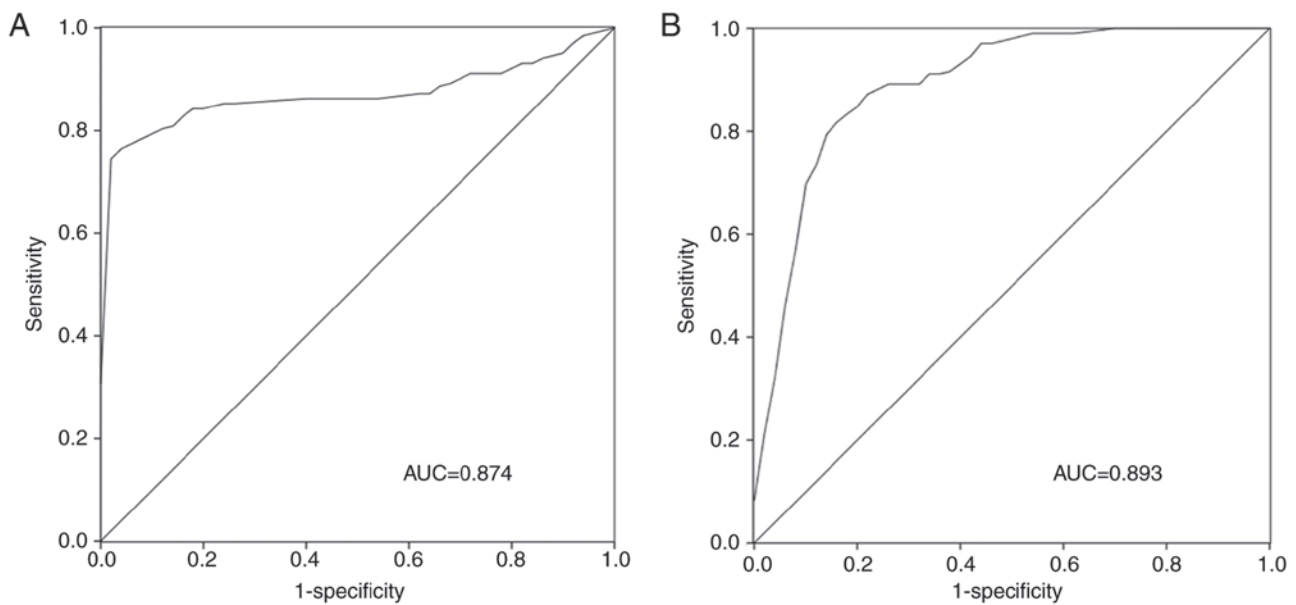


Figure 2. Receiver operating characteristic curves for NS patients based on PCT concentration and miR-181a expression. (A) The AUC of PCT was 0.874 and (B) the AUC of miR-181a expression was 0.893. NS, neonatal sepsis; miR, microRNA; AUC, area under the curve; PCT, procalcitonin.

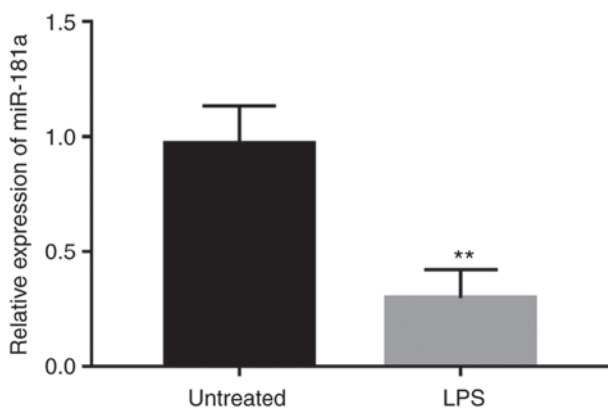


Figure 3. Relative expression of miR-181a in the LPS-treated monocytes. LPS stimulation in the primary monocytes led to a decrease in the expression of miR-181a. ** $P < 0.01$ vs. Untreated. miR, microRNA; LPS, lipopolysaccharide.

regulatory role of miR-181a on inflammation in monocytes. As presented in Fig. 5, the concentration of TNF- α and IL-8 was increased after LPS stimulation (all $P < 0.05$). Following modification of miR-181a levels in the monocytes, it was observed that overexpression of miR-181a resulted in decreased levels of TNF- α and IL-8, while inhibition of miR-181a led to increased concentrations of these two cytokines in the presence of LPS (all $P < 0.05$).

Discussion

The present study focused on the expression and clinical significance of miR-181a in patients with NS and explored the effects of miR-181a on LPS-induced inflammation in monocytes. RT-qPCR indicated that the serum levels of miR-181a were significantly downregulated in patients with NS compared with those in the controls, which may be of diagnostic value with considerable sensitivity and specificity. In

the monocytes extracted from the serum of patients with NS, the expression of miR-181a was also downregulated after LPS stimulation. TLR4 has been previously reported to be a target gene of miR-181a in immune thrombocytopenia (17), and in the present study, it was demonstrated that miR-181a directly inhibits the expression of TLR4 in monocytes. Furthermore, overexpression of miR-181a led to inhibition of LPS-induced inflammation, as evidenced by the decreased TNF- α and IL-8 concentrations.

Numerous studies have indicated the pivotal roles of miRNAs in the initiation and development of various human diseases, including malignancies (22), metabolic diseases (23) and cardiovascular diseases (24). In sepsis, there are also functional miRNAs that are linked to the progression of the disease by the regulation of inflammatory response, such as miR-150 (25) and miR-27a (26), e.g. miR-375 (25) and miR-25 (26). In addition, certain miRNAs with ectopic expression patterns have critical roles in the pathogenesis of NS by regulating the inflammatory response. For instance, miR-15a/16 has been reported to be upregulated in serum samples of patients with NS and may be involved in the inflammatory response in this disease (18). The expression of miR-132 and miR-223 was demonstrated to be downregulated in patients with NS compared with that in healthy infants and was associated with the expression of immune-associated genes involved in the TLR signaling pathway (27). In the present study, NS patients were recruited to estimate the expression of miR-181a. This study enrolled neonates with respiratory infection or pneumonia as controls but did not include healthy neonates. Firstly, blood samples were difficult to obtain from healthy individuals for ethical considerations; however, neonates with infections, but not sepsis, already underwent blood collection and examination. Thus, their blood samples were available with the approval from the families. Additionally, infections in neonates with pneumonia/respiratory tract infection contribute to the occurrence of NS (28).

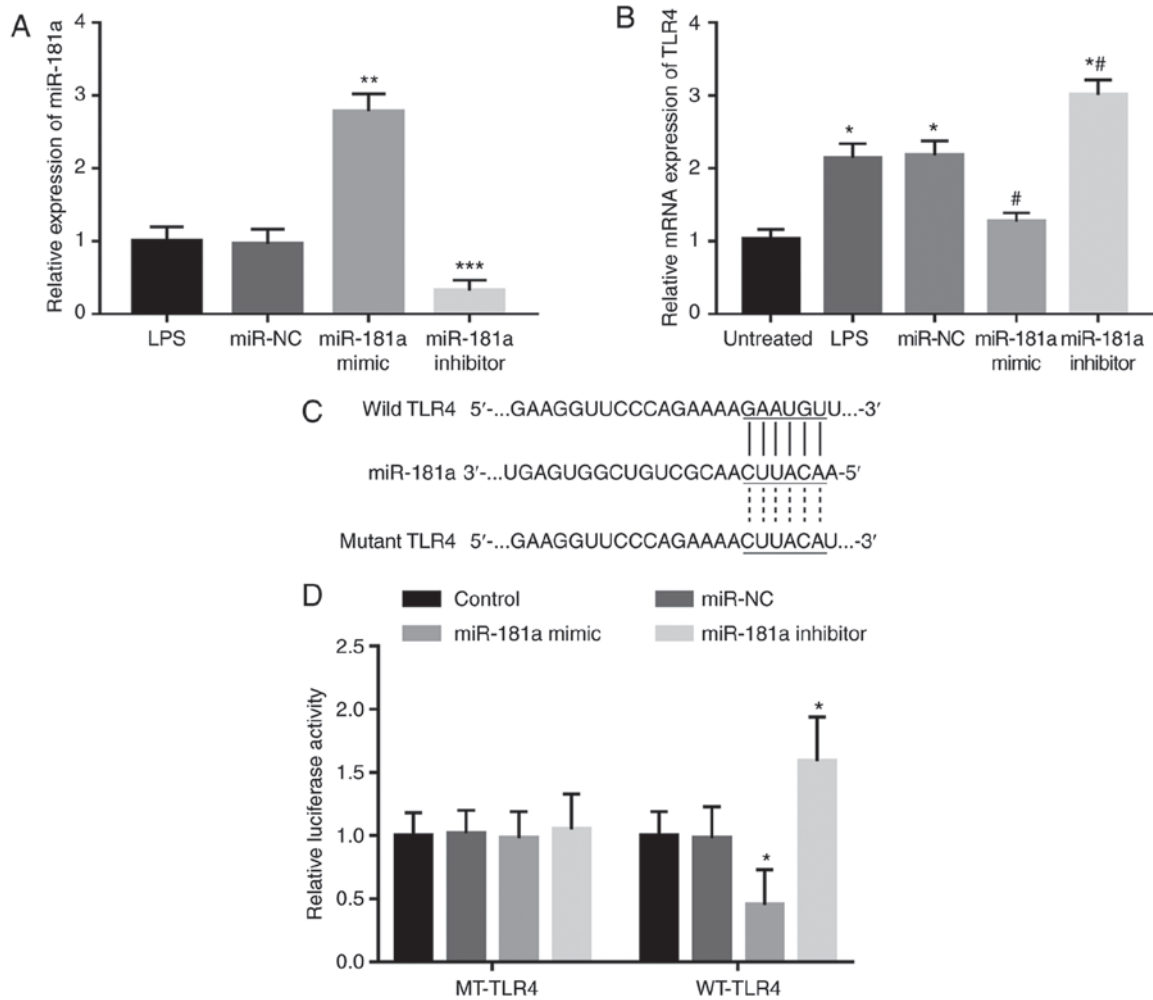


Figure 4. miR-181a directly inhibits the expression of TLR4 in primary monocytes. (A) The expression of miR-181a was increased by miR-181a mimics but was decreased by miR-181a inhibitor (** $P < 0.01$, *** $P < 0.001$ vs. LPS). (B) The increase in TLR4 induced by LPS was suppressed by overexpression of miR-181a but was promoted by the reduction of miR-181a in the monocytes (* $P < 0.05$ vs. Untreated; # $P < 0.05$ vs. LPS). (C) Complementary sequence of miR-181a in the 3'-UTR of TLR4. (D) Luciferase activity of the luciferase reporter plasmid carrying the WT 3'-UTR sequence of TLR4 was inhibited by overexpression of miR-181a, but was enhanced by inhibition of miR-181a (* $P < 0.05$ vs. Control). miR, microRNA; LPS, lipopolysaccharide; MT, mutant; WT, wild-type; TLR, Toll-like receptor; NC, negative control; UTR, untranslated region.

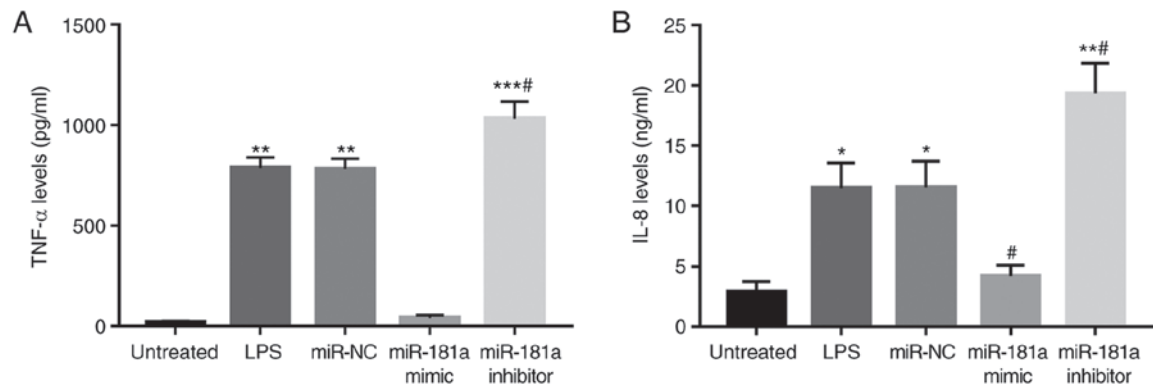


Figure 5. Effects of miR-181a on LPS-induced inflammation. LPS stimulation in the monocytes led to increased levels of (A) TNF- α and (B) IL-8, but these increases were suppressed by overexpression of miR-181a and were promoted by inhibition of miR-181a. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ vs. Untreated; # $P < 0.05$ vs. LPS. miR, microRNA; LPS, lipopolysaccharide; TNF, tumor necrosis factor; IL, interleukin; NC, negative control.

Thus, our study data may provide a diagnostic biomarker to screen the NS cases from the infection cohort. The expression analysis data shown a significant decrease in the expression

of miR-181a in the serum specimens of patients with NS compared with the controls. In a study by Chen *et al* (16), downregulated expression of circulating miR-181a was also

reported in patients with NS. Thus, miR-181a may have a pivotal role in the progression of NS.

Accurate diagnosis is the first and most important prerequisite of the efficient treatment of diseases. To improve the treatment of infectious diseases in neonates, it is of value to screen NS cases from the neonates with pneumonia/respiratory tract infection. CRP, WBC and PCT are the established and widely used diagnostic biomarkers for NS, but their application has limited specificity (27). Previous studies indicated that infection with bacteria, fungi or parasites may lead to increases in WBC and the levels of CRP and PCT (29). Thus, elevated WBC, CRP or PCT may be detected in certain infectious and inflammatory diseases other than NS, including pneumonia (30) and respiratory infection (31). In the present study, patients with NS and neonates with respiratory infection or pneumonia as the control group were enrolled. No significant differences were observed in age, sex and body weight between the two groups, but the cases with NS had significantly higher PCT levels than the controls. Regarding the CRP and WBC count, the difference did not reach statistical significance, although their values were increased in the NS group compared with that in the controls, which may be due to the increased inflammatory responses induced by respiratory infection in the control neonates. Thus, in addition to the detection of biological biomarkers, the clinical manifestations, as well as further verification from bacterial cultures, is essential for the diagnosis of NS.

miRNAs are considered ideal diagnostic tools for various human diseases, which is mainly due to their specific expression patterns and stability in blood samples (32). For instance, downregulated expression of miR-124 in the serum was previously described as a diagnostic biomarker for patients with osteosarcoma (33). Increased serum levels of miR-155-5p and miR-133a-3p were proven to serve as diagnostic and prognostic biomarkers in patients with sepsis (34). In patients with NS, upregulated expression of miR-15a/16 was also determined to be a diagnostic biomarker (18). Given the markedly decreased expression of miR-181a in the serum samples of patients with NS, an ROC analysis was performed in the present study, demonstrating that aberrant expression of miR-181a may serve as a diagnostic biomarker to screen NS patients from the neonates with pneumonia/respiratory tract infection, with relatively high sensitivity and specificity. At present, the available diagnostic methods for NS are limited by their poor sensitivity or specificity. The present study may provide novel and efficient diagnostic biomarkers for patients with NS.

NS is characterized by inflammatory responses. TLR4 is a protein that has a key role in the innate immune system (35). TLR4 is sensitive to external signals and is an important mediator for inflammatory events (36). He *et al.* (17) reported that TLR4 is a target gene of miR-181a in immune thrombocytopenia. Thus, the present study also focused on the effect of miR-181a on the expression of TLR4 in monocytes collected from the blood samples of NS patients. The results indicated that TLR4 was a target gene of miR-181a and was downregulated by overexpression of miR-181a. Furthermore, the role of miR-181a in the regulation of inflammatory cytokines was explored in monocytes treated with LPS. The LPS-induced increases in the levels of inflammatory cytokines were all suppressed following overexpression of

miR-181a, indicating the suppressive role of miR-181a in the regulation of LPS-induced inflammation. Collectively, it may be indicated that miR-181a may inhibit the LPS-induced inflammatory response by downregulation of TLR4. Although the present study provided evidence for the regulatory effect of miR-181a on the expression of TLR4, further research is required to confirm this interaction and investigate the effects of miR-181a on TLR4-associated signaling. Autophagy, which can be regulated by TLR4, has been reported to be involved in the progression of sepsis (37). Interestingly, previous studies have found a regulatory role for miR-181a on autophagy in the pathogenesis of some diseases, such as myocardial hypertrophy (38) and gastric cancer (39). Thus, it was deduced that the miR-181a/TLR4 axis might also be involved in the regulation of autophagy in NS development. However, this hypothesis was not investigated in the present study, which is one of the limitations of this present study. Additionally, the accuracy of the clinical research data may be limited by the small sample size and further investigations with larger research cohorts are required.

In conclusion, the present study revealed that the serum expression of miR-181a is downregulated in patients with NS and the dysregulation of miR-181a serves as a candidate diagnostic biomarker for NS. Overexpression of miR-181a in monocytes was able to improve the LPS-induced inflammatory reaction by targeting TLR4, which may further uncover the pathologic mechanisms of action underlying the development of NS.

Acknowledgements

Not applicable.

Funding

No funding was received.

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

GL made substantial contributions to the conception and design of the study, analysis and interpretation of data and revision of the manuscript. WL and JG were involved in the acquisition of data and drafting of the manuscript. All authors gave final approval of the version to be published.

Ethics approval and consent to participate

The experimental protocols were approved by the Ethics Committee of Yidu Central Hospital of Weifang (Weifang, China) and written informed consent was obtained from the families of the patients.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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