

MicroRNA-20a promotes inflammation via the nuclear factor- κ B signaling pathway in pediatric pneumonia

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Abstract. Pneumonia is a common respiratory disease worldwide, which is preventable and treatable; however, it is recognized as a leading cause of mortality in children. The present study aimed to investigate the role and mechanism of microRNA (miR)-20a in inflammation in pediatric pneumonia. Clinical serum samples were collected from children with pneumonia and healthy children. Initially, the serum expression levels of miR-20a were detected by reverse transcription-quantitative polymerase chain reaction. Subsequently, A549 cells were randomly divided into four groups: Control group; lipopolysaccharide (LPS; 1 μ g/ml) group; LPS + miR-20a group; and LPS + miR-20a + pyrrolidine dithiocarbamate (PDTC; 100 mmol/l) group. The concentrations of interleukin-6 (IL-6), tumor necrosis factor (TNF)- α and C-reactive protein (CRP) in clinical serum samples and A549 cells were determined by ELISA. In addition, the protein expression levels of inhibitor of nuclear factor (NF)- κ B α (I κ B α) and phosphorylated (p)-NF- κ B were measured by western blotting. The results demonstrated that miR-20a was upregulated in children with pneumonia and in lung cells with LPS-induced inflammatory injury ($P < 0.01$). In addition, compared with the LPS group, cells in the LPS + miR-20a group exhibited increased expression levels of IL-6, TNF- α and CRP ($P < 0.05$). Overexpression of miR-20a also resulted in upregulation of the expression levels of I κ B α and p-NF- κ B compared with in the LPS group ($P < 0.05$). Furthermore, treatment with the NF- κ B inhibitor PDTC inhibited the expression of inflammatory factors compared with in the LPS + miR-20a group ($P < 0.05$). In conclusion, the present study indicated that

miR-20a is upregulated in pediatric pneumonia, and overexpression of miR-20a may promote inflammation through activation of the NF- κ B signaling pathway.

Introduction

Pneumonia is a common respiratory disease worldwide, which is preventable and treatable (1); however, it is recognized as a leading cause of mortality in children (2). It has been reported that between 1.1 and 1.4 million children succumb to pneumonia annually (3). Due to poor health care or incomplete treatment, pediatric pneumonia often recurs, which can result in numerous severe complications and a poor prognosis, thereby affecting the child's development, even leading to mortality (4,5). Although the prevalence of pediatric mortality has been reduced due to recent surgical and medical advances, pediatric pneumonia remains at a high risk of recurrence and hospitalization (6,7). Therefore, it is essential to investigate the underlying pathogenesis and to identify effective therapeutic targets for the treatment of pediatric pneumonia.

MicroRNAs (miRNAs/miRs) are a group of small non-coding RNAs, between 19 and 24 nucleotides long, which regulate the expression of target genes by binding with complementary sites on target 3'-untranslated region (3'UTR) (8). miRNAs have been reported to be implicated in the majority of cellular processes, including cell proliferation, apoptosis, migration, differentiation and metabolism, as well as in innate immunity, inflammation and infection (9,10). Previous studies have demonstrated that miRNAs serve potential roles in some types of cancer, through regulating gene expression levels and cancer pathogenesis-associated pathways, including in colorectal cancer (11), chronic lymphocytic leukemia (12), breast cancer (13), hepatocellular carcinoma (14) and lung cancer (15).

In addition to the effects of miRNAs on cancer, much attention has been paid to the potential roles of miRNAs in pediatric lung development (16) and respiratory diseases (17). A previous study suggested that numerous miRNAs associated with inflammation (miR-132, miR-181a, miR-221 and miR-222) may serve a role in the pathogenesis of *Streptococcus pneumoniae* meningitis (18). Recently, miR-20a has been reported to be upregulated and participate in the regulation of cell proliferation in lung cancer (19). However, it is unclear

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whether miR-20a is associated with the pathogenesis of pediatric pneumonia.

The present study evaluated the expression levels of miR-20a in children with pneumonia and in a cell model of lipopolysaccharide (LPS)-induced inflammatory lung injury (20). Subsequently, the role and mechanisms of miR-20a in pediatric pneumonia-associated inflammation were investigated.

Materials and methods

Patients. A total of 16 children (8 male and 8 female; mean age \pm standard deviation, 11.4 ± 2.16 years; age range, 8-15) with pneumonia were recruited to the present study. Pneumonia was diagnosed according to clinical presentation, respiratory symptoms, bacterial infection and chest X-ray. In addition, 16 gender-matched children (mean age \pm standard deviation, 11.3 ± 2.24 years; age range, 7-15) with fever were included as the control group; these children were treated in the Emergency Department and had no respiratory symptoms. These 32 patients were recruited from the Jinan Maternity and Child Care Hospital (Jinan, China) between June 2012 and December 2013. None of the patients underwent any treatment prior to blood collection. Venous blood samples (5 ml) were collected from the patients and the present study was approved by the Ethics Committee of the Jinan Maternity and Child Care Hospital. Written informed consent was obtained from the legal guardians of all participants.

Cell culture and treatment. The A549 human lung adenocarcinoma cell line was purchased from the Shanghai Cell Bank of Chinese Academy of Sciences (Shanghai, China). Cells were cultured in RPMI 1640 medium (Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA) supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin (both Beijing Transgen Biotechnology Co., Ltd., Beijing, China) at 37°C in an incubator containing 5% CO₂. A total of 2 ml cells/well, at a concentration of 5×10^5 cells/ml, were cultured in a 6-well plate. Subsequently, cells were separated into groups, and were treated with LPS (Sigma-Aldrich; Merck KGaA, Darmstadt, Germany), miR-20a mimic (sense 5'-UAA AGUGCUUAUAGUGCAGGUAG-3' and antisense 5'-ACC UGCACUAUAAGCACUUUAUU-3'), mimic control (sense 5'-UGC UUAGUAUAAGAUGCAGGUAG-3' and antisense 5'-ACGCUAUAUCUUUAUAGCACACUU-3') (both Shanghai GenePharma Co., Ltd., Shanghai, China) or the nuclear factor (NF)- κ B inhibitor pyrrolidine dithiocarbamate (PDTC; Sigma-Aldrich; Merck KGaA). Briefly, the cells were divided into the following four groups: Control group (non-treated cells); LPS group, in which cells were stimulated with LPS (1 μ g/ml) for 12 h at 37°C to induce inflammatory lung cell injury (20); LPS + miR-20a group, in which cells overexpressing miR-20a were treated with LPS (1 μ g/ml) for 12 h at 37°C; and LPS + miR-20a + PDTC group, in which cells overexpressing miR-20a were treated with PDTC (100 mmol/l) for 30 min at 37°C and then treated with LPS (1 μ g/ml) for 12 h at 37°C (21). In addition, an LPS + mimic control group, in which cells were transfected with mimic control and then treated with LPS (1 μ g/ml) for 12 h at 37°C, was generated. To overexpress miR-20a, the miR-20a mimic (50 nM) was transfected

Table I. Primer sequences for specific genes.

Gene	Primer	Sequence (5'-3')
miR-20a	Forward	GCCGCGCTAAAGTGCTTATAGTG
	Reverse	CACCAGGGTCCGAGGT
U6	Forward	TGCGGGTGCTCGCTTCGGCAGC
	Reverse	CCAGTGCAGGGTCCGAGGT
miR-20a, microRNA-20a.		

into A549 cells (1×10^5 cells) using 20 μ l Lipofectamine 2000 (Invitrogen; Thermo Fisher Scientific, Inc.) following the manufacturer's protocol. Transfection with the mimic control was the same as the transfection of miR-20a. Transfected cells were harvested at 48 h post-transfection for subsequent experiments.

ELISA analysis. Blood samples were collected from the children and were centrifuged at $1,500 \times g$ for 10 min at 4°C. The supernatant serum samples were then collected and stored at -80°C. The serum concentrations of interleukin (IL)-6 (cat. no. S6050), tumor necrosis factor (TNF)- α (cat. no. STA00C) and C-reactive protein (CRP; cat. no. SCRPO0) were determined using corresponding ELISA kits (R&D Systems, Inc., Minneapolis, MN, USA) according to the manufacturer's protocols. The absorbance was measured at 450 nm using a microplate reader (Thermo Fisher Scientific, Inc.). The concentrations of IL-6, TNF- α and CRP were calculated based on the standard curve.

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR) analysis. Total RNA was extracted from blood samples using the RNAprep pure Blood kit (Tiangen Biotech, Beijing, China) and total RNA was extracted from treated cells using TRIzol reagent (Invitrogen; Thermo Fisher Scientific, Inc.). Subsequently, RNA was measured using a UV spectrophotometer (BD Biosciences, San Diego, CA, USA) at A₂₆₀, and cDNA was generated from miRNAs using stem-loop RT primers (Applied Biosystems; Thermo Fisher Scientific, Inc.) with the following cycling parameters: 30°C for 10 min, followed by 42°C for 30 min and 95°C for 5 min. Primer sequences for miR-20a and U6 small nuclear (sn)RNA are presented in Table I. SYBR® *Premix Ex Taq*TM kit (Takara Biotechnology Co., Ltd., Dalian, China) was used to perform PCR amplification; the PCR program was as follows: 94°C for 10 min, followed by 40 cycles at 94°C for 15 sec, 56°C for 30 sec and 72°C for 1 min, and finally 72°C for 10 min. U6 snRNA was used as an internal control. Relative quantification and calculations were conducted using the quantification cycle (Cq) method ($2^{-\Delta\Delta Cq}$) (22).

Western blotting. The cells were collected and the proteins were extracted using RIPA buffer (Beyotime Institute of Biotechnology, Shanghai, China) containing a cocktail of protease inhibitors (Roche Diagnostics, Basel, Switzerland). Bicinchoninic acid protein assay kit (Sangon Biotech Co., Ltd., Shanghai, China) was used to determine protein concentration.

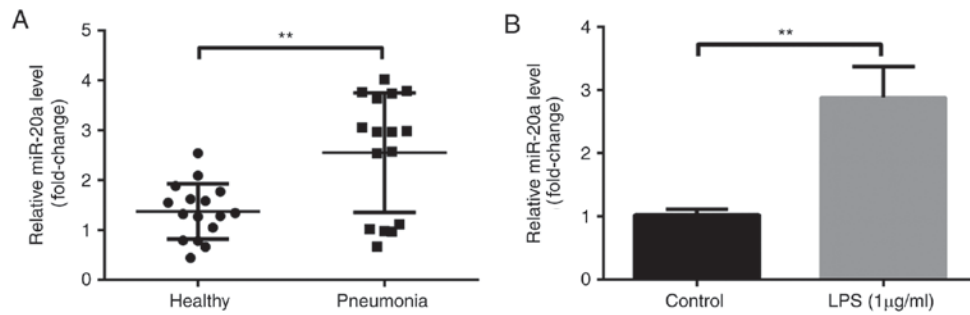


Figure 1. miR-20a was upregulated in children with pneumonia and in A549 cells with LPS-induced inflammatory injury. (A) Expression levels of miR-21 in children with pneumonia and in healthy children (n=16/group). (B) Expression levels of miR-21 in A549 cells treated with or without LPS (1 µg/ml). Data are presented as the mean ± standard deviation. **P<0.01. LPS, lipopolysaccharide; miR, microRNA.

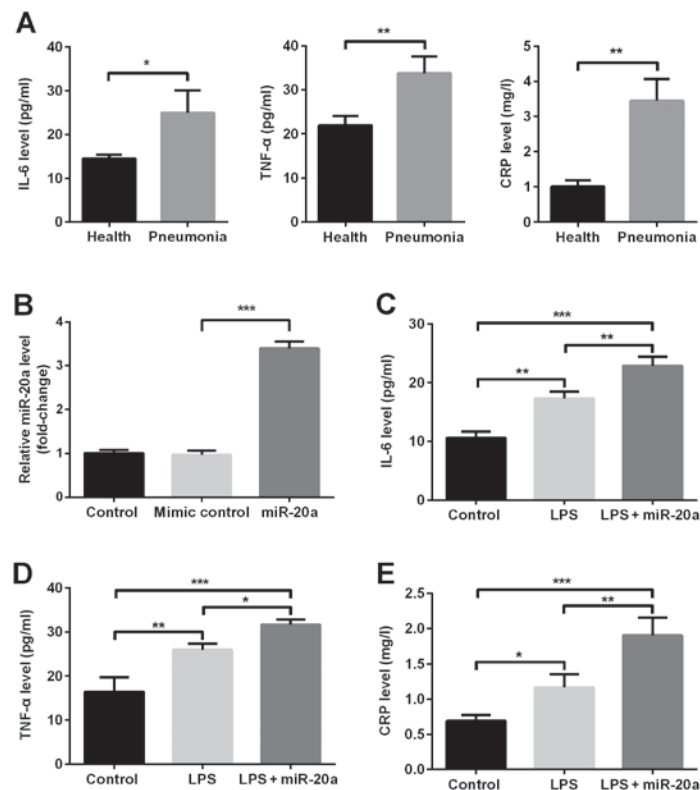


Figure 2. miR-20a promotes the expression of inflammatory factors, including IL-6, TNF-α and CRP, in A549 cells with LPS-induced inflammatory injury. (A) Concentrations of IL-6, TNF-α and CRP in children with pneumonia and in healthy children (n=16/group). (B) Expression levels of miR-20a in transfected A549 cells; and the concentrations of (C) IL-6, (D) TNF-α and (E) CRP in A549 cells. In addition, an LPS + mimic control group was generated; the results from this group were not significantly different compared with the LPS group; therefore, data are not shown. Data are presented as the mean ± standard deviation. *P<0.05; **P<0.01; ***P<0.001. CRP, C-reactive protein; IL-6, interleukin-6; LPS, lipopolysaccharide; miR, microRNA; TNF-α, tumor necrosis factor-α.

Subsequently, equal amounts of protein (30 µg/lane) were separated by 10% SDS-PAGE and were transferred to a polyvinylidene fluoride membrane. After blocking with 5% non-fat milk at room temperature for 2 h, the membrane was incubated with rabbit anti-human inhibitor of NF-κB α (IκBα; cat. no. SAB4501994; 1:500), phosphorylated (p)-NF-κB (cat. no. SAB4301496; 1:500) polyclonal antibodies and β-actin monoclonal antibody (cat. no. SAB2100037; 1:1,000) (all Sigma-Aldrich; Merck KGaA) at 4°C overnight. β-actin was used as an internal control. After washing with PBS, the membrane was incubated with horseradish peroxidase-conjugated goat anti-rabbit secondary antibody (cat. no. 31460; 1:10,000; Thermo Fisher Scientific, Inc.) for 2 h at room

temperature. The membrane was washed again with PBS and color was developed using a 3,3'-diaminobenzidine substrate (Sangon Biotech Co., Ltd.). Image Pro Plus software (version 5.0; Media Cybernetics, Inc., Rockville, MD, USA) was used to semi-quantify the expression levels of the target proteins.

Statistical analysis. All data are presented as the mean ± standard deviation, and SPSS 16.0 statistics software (SPSS, Inc., Chicago, IL, USA) was used to analyze the data. All data were initially tested for Gaussian distribution. Subsequently, data were analyzed by unpaired two-tailed t-test (for two groups) or one-way analysis of variance followed by a Tukey post hoc test (for more than two groups).

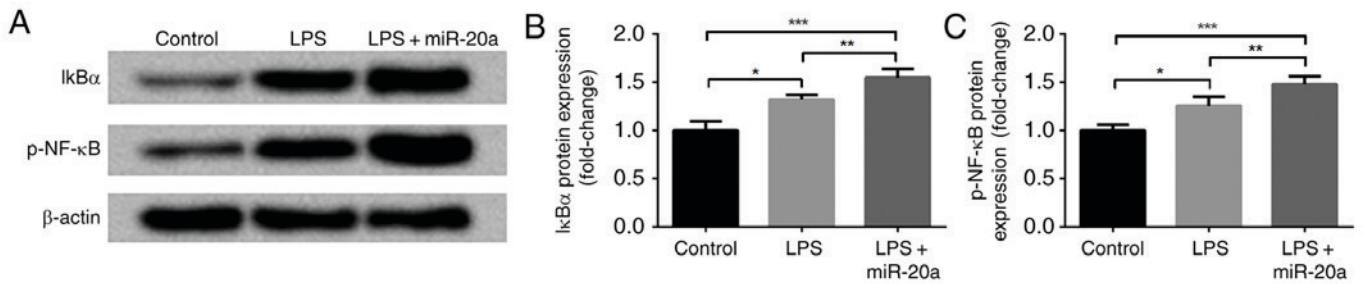


Figure 3. miR-20a enhances the expression levels of NF-κB signaling pathway-associated proteins, including IκBα and p-NF-κB, in A549 cells with LPS-induced inflammatory injury. (A) IκBα and p-NF-κB expression was detected using western blotting; and the protein expression levels of (B) IκBα and (C) p-NF-κB were semi-quantified. In addition, an LPS + mimic control group was generated; the results from this group were not significantly different compared with the LPS group; therefore, data are not shown. Data are presented as the mean + standard deviation. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$. IκBα, inhibitor of NF-κB α; LPS, lipopolysaccharide; miR, microRNA; NF-κB, nuclear factor-κB; p-NF-κB, phosphorylated-NF-κB.

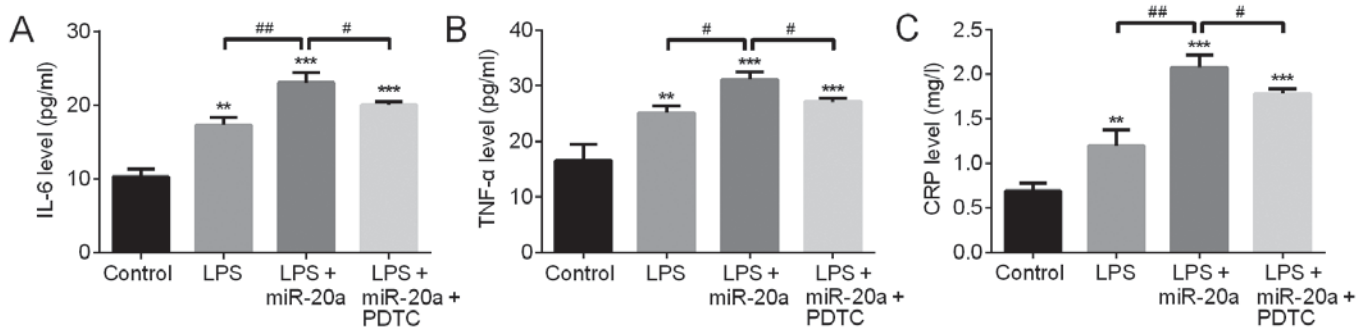


Figure 4. NF-κB inhibitor PDTC inhibits the proinflammatory role of miR-20a in A549 cells with LPS-induced inflammatory injury. The expression levels of (A) IL-6, (B) TNF-α and (C) CRP were detected by ELISA analysis. In addition, an LPS + mimic control group was generated; the results from this group were not significantly different compared with the LPS group; therefore, data are not shown. Data are presented as the mean ± standard deviation. ** $P < 0.01$, *** $P < 0.001$ vs. control group. * $P < 0.05$, ** $P < 0.01$ vs. LPS + miR-20a group. CRP, C-reactive protein; IL-6, interleukin-6; LPS, lipopolysaccharide; miR, microRNA; PDTC, pyrrolidine dithiocarbamate; TNF-α, tumor necrosis factor-α.

$P < 0.05$ was considered to indicate a statistically significant difference.

Results

miR-20a expression in patients with pneumonia and in LPS-induced cells. To explore the expression levels of miR-20a in patients with pneumonia, an RT-qPCR analysis was performed; the results demonstrated that miR-20a expression was higher in patients with pneumonia compared with in healthy controls ($P < 0.01$; Fig. 1A). In addition, LPS was used to induce inflammatory injury in A549 cells. Compared with in the control group, the expression levels of miR-20a were increased in the LPS group ($P < 0.01$; Fig. 1B).

Effects of miR-20a overexpression on inflammation in pneumonia. The present study investigated the association between miR-20a and the inflammatory reaction. ELISA analysis revealed that the serum concentrations of inflammatory factors, including IL-6, TNF-α and CRP, were higher in patients with pneumonia compared with in healthy controls ($P < 0.05$; Fig. 2A). The results of an RT-qPCR analysis indicated that the expression levels of miR-20a were significantly upregulated following transfection of cells with miR-20a mimic compared with in the mimic control group

($P < 0.001$; Fig. 2B), confirming that miR-20a was successfully overexpressed. Subsequent experiments demonstrated that the concentrations of inflammatory factors, including IL-6, TNF-α and CRP, were increased in the LPS group compared with in the control group ($P < 0.05$; Fig. 2C-E). In addition, overexpression of miR-20a further increased the concentrations of IL-6, TNF-α and CRP compared with the LPS group ($P < 0.05$). The levels of inflammatory cytokines in the LPS + mimic control group were not significantly different compared with the LPS group (data not shown).

Effects of miR-20a overexpression on the NF-κB signaling pathway in LPS-induced cells. To further investigate the mechanism underlying the effects of miR-20a on the induction of inflammation, NF-κB signaling pathway-associated proteins, including IκBα and p-NF-κB, were measured by western blotting. As shown in Fig. 3A-C, the protein expression levels of IκBα and p-NF-κB were increased in the LPS group compared with in the control group ($P < 0.05$). The expression of total NF-κB was consistent with that of β-actin (data not shown). In addition, the expression levels of IκBα and p-NF-κB were further increased in the LPS + miR-20a group compared with in the LPS group ($P < 0.05$). The expression levels of IκBα and p-NF-κB in LPS + mimic control group were not significantly different compared with the LPS group (data not shown).

Effects of the NF- κ B inhibitor PDTC on miR-20a-induced inflammation in LPS-treated cells. To evaluate the role of the NF- κ B signaling pathway in miR-20a-induced inflammation, the NF- κ B inhibitor PDTC was used to suppress the NF- κ B signaling pathway. ELISA was subsequently used to detect the expression levels of inflammatory factors. The results demonstrated that following treatment with the NF- κ B inhibitor PDTC, the expression levels of IL-6, TNF- α and CRP were markedly decreased compared with in the LPS + miR-20a group ($P < 0.05$; Fig. 4). The levels of inflammatory cytokines in the LPS + mimic control group were not significantly different compared with the LPS group (data not shown).

Discussion

miRNAs have been reported to have a role in inflammatory diseases (23). The results of the present study demonstrated that the expression levels of miR-20a were increased in children with pneumonia and in lung cells with LPS-induced inflammatory injury. Furthermore, the results indicated that miR-20a overexpression may promote the expression of inflammatory factors, including IL-6, TNF- α and CRP. miR-20a overexpression also increased the expression levels of NF- κ B signaling pathway-associated proteins, including I κ B α and p-NF- κ B, whereas treatment with the NF- κ B inhibitor PDTC was able to inhibit the enhancing effects of miR-20a on the inflammatory reaction.

It has previously been demonstrated that miRNAs exert marked regulatory effects on inflammation, and various miRNAs have been identified and considered to participate in the pathological mechanisms of inflammatory diseases (24). Bazzoni *et al* (25) reported that in neutrophils and macrophages, miR-9 overexpression could be induced by LPS and negatively regulated NF- κ B signaling pathway-dependent inflammatory responses. miR-21 has also been identified to target tumor suppressor genes and negatively regulate the LPS-stimulated Toll-like receptor (TLR)4 pathway, which may result in activation of NF- κ B and production of IL-10 (26). Downregulated miR-125b has been indicated to promote the expression of TNF- α in LPS-induced activated macrophages (27). Furthermore, miR-155 has been reported to suppress the inflammatory reaction through regulating the expression of myeloid differentiation primary response protein 88, which serves an important role in the TLR pathway (28). Taganov *et al* (29) revealed that miR-146 may be significantly upregulated in human monocytic cells stimulated by LPS, and miR-146 was able to inhibit the expression of target genes, including IL-1 receptor-associated kinase 1 and TNF receptor-associated factor 6, and negatively regulate TLR signaling. It was also demonstrated that miR-146a was upregulated in monocytes stimulated by inflammatory cytokines, including TNF- α and IL-1 β (29). Following activation of the TLR pathway, miR-147 has been reported to be upregulated and to inhibit the excessive inflammatory responses in murine lung macrophages (30).

The present study focused on the role of miR-20a in pediatric pneumonia. miR-20a is a member of the miR-17-92 cluster, which is involved in tumorigenesis (31). Previous studies have demonstrated that miR-20a is associated with cell proliferation (32) and cell cycle progression (33) by regulating

the expression of transcription factor E2F1. Fan *et al* (34) also reported that miR-20a overexpression was able to promote cell proliferation and invasion by inhibiting the expression of targeted amyloid precursor protein in ovarian cancer cells. Furthermore, it has been demonstrated that miR-20 serves an important role in inflammatory responses. Philippe *et al* (35) reported that miR-20 could effectively inhibit the production of inflammatory cytokines, including IL-6, C-X-C motif chemokine ligand 10, IL-1 β and TNF- α , in LPS-activated fibroblast-like synoviocytes. Conversely, miR-20a was reported to promote macrophage inflammatory responses through modulating the expression of signal-regulatory protein α both *in vitro* and *in vivo* (36). The various effects of miR-20 on the inflammatory response may be associated with the different target genes of miR-20 in various cell types. In the present study, a high expression of miR-20a was detected in pediatric pneumonia, and the results revealed that miR-20a overexpression increased the expression levels of IL-6, TNF- α and CRP in lung cells with LPS-induced inflammatory injury. These findings indicated that miR-20a may be involved in the inflammatory response in pediatric pneumonia.

The present study also investigated the mechanism underlying the effects of miR-20a on inflammation in pediatric pneumonia. The NF- κ B signaling pathway is considered closely associated with inflammation; the activation of NF- κ B induces the secretion of proinflammatory cytokines, including IL-1 and TNF- α , chemokines and adhesion molecules. Previous studies have demonstrated that NF- κ B is activated in numerous inflammatory disorders, including arthritis (37), glomerulonephritis (38), gastritis (39) and asthma (40). The interaction between miRNAs (miR-21, miR-146, miR-155, miR-181b and miR-301a) and NF- κ B activation has been demonstrated in human disease (41). Bhaumik *et al* (42) demonstrated that NF- κ B activity and the inflammatory pathway can be negatively regulated by miR-146a/b in breast cancer cells. Notably, the results of the present study preliminarily confirmed that the NF- κ B signaling pathway was activated by miR-20a overexpression, and the proinflammatory role of miR-20a could be inhibited following treatment of LPS-induced cells with an NF- κ B inhibitor. These results suggested that miR-20a may promote inflammation by activating the NF- κ B signaling pathway.

In conclusion, the present study confirmed that miR-20a was upregulated in children with pediatric pneumonia and in human lung adenocarcinoma A549 cells with LPS-induced inflammatory injury. In addition, overexpression of miR-20a was able to promote inflammation, which may be associated with the NF- κ B signaling pathway in pediatric pneumonia. However, further studies are required to investigate the target molecules of miR-20a in pediatric pneumonia.

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