Overexpression of miR-874-3p alleviates LPS-induced apoptosis and inflammation in alveolar epithelial cell by targeting EGR3/NF-κB

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Abstract

Objective: MicroRNA (miRNA) is implicated in the pathogenic mechanism of pneumonia. Role of miR-874-3p in pediatric pneumonia was therefore evaluated in this study. Methods: Expression levels of miR-874-3p in the serum samples from pediatric patients with pneumonia and LPS-treated HPAEpiC were determined by RT-qPCR (reverse transcription quantitative real-time PCR). Secretion of inflammatory factors in LPS-treated HPAEpiC were determined by qRT-PCR and ELISA. Cell viability and apoptosis were evaluated by CCK8 and flow cytometry, respectively. HPAEpiC was used for the validation of binding target of miR-874-3p. Mechanism was determined by NF-κB promoter activity assay. Results: MiR-874-3p was reduced in serum samples of pediatric patients with pneumonia, and LPS treatment dose-dependently decreased miR-874-3p expression in HPAEpiC. TNF-α and IL-1β expression levels were increased in HPAEpiC post LPS treatment. Over-expression of miR-874-3p attenuated LPS-induced increase of TNF-α and IL-1β and reversed LPS-induced decrease of cell viability and increase of cell apoptosis in HPAEpiC. EGR3 (early growth response 3) was a target gene of miR-874-3p. EGR3 over-expression reversed miR-874-3p over-expression-induced increase of cell viability, decrease of cell apoptosis, TNF-α and IL-1β in LPS-induced HPAEpiC. Over-expression of miR-874-3p reduced p65 expression and NF-κB promoter activity in LPS-induced HPAEpiC, while EGR3 over-expression reversed these suppressive effects. Conclusion: MiR-874-3p negatively regulates EGR3 expression to promote cell viability and inhibit apoptosis as well as inflammation in LPS-treated HPAEpiC via suppression of NF-κB pathway, suggesting a potential therapeutic strategy for pneumonia.

Keywords: miR-874-3p, EGR3, LPS, HPAEpiC, pediatric pneumonia

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INTRODUCTION

Pneumonia is a common and frequently occurring respiratory disease in pediatrics. It is one of the three most important pediatric diseases and the leading cause of infant mortality worldwide (Alcoba et al., 2017). Pneumonia is associated with pathogenic factors post infection with bacteria or viruses, and microbial pathogenesis-induced stimulation of inflammatory response was considered as one of the causes of severe acute pediatric pneumonia (Qinge et al., 2019; Zhang et al., 2019). Alveolar epithelial cells are the major targets of inflammatory factors secreted by the infected epithelial cells during the inflammatory response in the lungs (Ito et al., 2015). Damage to alveolar epithelial cells is a key step in the development of pneumonia and ultimately leads to fluid imbalance (Guillot et al., 2013). The state of alveolar epithelial cells contributes to the loss of cellular defense during development of pneumonia (Robb et al., 2016). Therefore, it is important to reduce the inflammation and apoptosis of alveolar epithelial cells during the treatment of pediatric pneumonia.

Dysregulation of microRNAs (miRNAs) has been identified as diagnostic biomarkers for pneumonia (Huang et al., 2017), and miRNAs could mediate expression of target genes involved in immune response during pneumonia (Huang et al., 2018). Therefore, miRNAs were widely investigated in pediatric pneumonia. Recently, miRNAs have been reported to be implicated in apoptosis and inflammation of alveolar epithelial cells (Li & Liu, 2020). Suppression of apoptosis and inflammation in alveolar epithelial cells by miR-424 could ameliorate acute respiratory distress syndrome (Cheng et al., 2020). MiR-874-3p was reported to increase cell proliferation and suppress apoptosis during cerebral ischemia/reperfusion injury (Jiang et al., 2019), and inhibit inflammatory response during ischemic stroke (Xie et al., 2020). Given that the expression of miR-874-3p was down-regulated in patients with pneumonia in this study, we speculated that miR-874-3p might regulate apoptosis and inflammation of alveolar epithelial cells and play a certain regulatory role in pneumonia development.

EGR3 (early growth response 3) functions as a transcriptional factor to regulate genes involved in inflammation (Xie et al., 2020), cell proliferation (Li et al., 2012) and differentiation (Miao et al., 2017). EGR1 pathway was involved in lung inflammation (Cho et al., 2006), and epithelial barrier disruption (Choi et al., 2012). However, no researches about regulation of EGR in inflammation during pneumonia have not been reported yet. This study was performed to investigate whether miR-874-3p could target EGR3 to regulate apoptosis and inflammation of alveolar epithelial cells.
MATERIALS AND METHODS

Serum collection

This study was approved by the Ethics Committee of Zhejiang Zhoushan Putuo District Hospital of Traditional Chinese Medicine. Twenty-seven pediatric patients with pneumonia and nineteen healthy children were recruited at Zhejiang Zhoushan Putuo District Hospital of Traditional Chinese Medicine with written parental informed consents. Patients with anti-inflammatory therapy were excluded from this study, and patients with diagnostic criteria of pneumonia were included in this study. Serum samples were obtained from pneumonia patients and healthy children and stored at –80°C for the functional assays.

CELL CULTURE, TREATMENT AND TRANSFECTION

Human pulmonary alveolar epithelial cell (HPAEpiC; ATCC; Manassas, VA, USA) was cultured in RPMI-1640 medium with high glucose (Lonza, Basel, Switzerland) containing 10% fetal bovine serum (Gibco, Waltham, MA, USA) at 37°C. For cell transfection, mimic and inhibitor of miR-874-3p and negative controls (NC mimic and NC inhibitor) were purchased from GenePharma (Shanghai, China). Full length CDS of EGR3 was inserted into pcDNA3.1 vector (Invitrogen, Carlsbad, CA, USA). Transfection was then performed via Lipofectamine 2000 (Invitrogen) into HPAEpiC. Two days after transfection, HPAEpiC was incubated in RPMI-1640 medium with high glucose containing 10% fetal bovine serum and 1, 10 or 50 mg/L LPS (Sigma Aldrich, St. Louis, MO, USA) for 48 hours before functional assays.

Detection of TNF-α and IL-1β

Supernatant was harvested from medium of cell culture through centrifugation at 400×g for 10 minutes. Commercial Elisa kits (Thermo Fisher, Waltham, MA, USA) were used for the detection of TNF-α and IL-1β.

Cell viability and apoptosis assays

HPAEpiC with indicated treatment was seeded for 24 hours followed by LPS incubation. CCK8 solution (Dojindo, Tokyo, Japan) was then added to the wells and incubated at 37°C for 1 hour. Absorbance at 450 nm was measured by Microplate Autoreader (Thermo Fisher, Waltham, MA, USA) with PI (100 µg/mL) and 1 U/ml ribonuclease (Invitrogen) for 30 minutes, and then incubated with fluorescein isothiocyanate-conjugated annexin V (20 µg/mL) for another 20 minutes. Apoptosis was analyzed by FACS flow cytometer (Attune, Life Technologies, Darmstadt, Germany).

Luciferase reporter assay

3′-UTR of EGR3 or sequence with mutation at the potential binding site between EGR3 and miR-874-3p were subcloned into pGL3 luciferase vector (Promega, Madison, Wisconsin, USA). Co-transfection with EGR3-WT/EGR3-MUT or mimic and inhibitor of miR-874-3p were performed through Lipofectamine 2000. Dual Luciferase Assay Kit (Promega) was used to detect luciferase activities two days later.

NF-κB promoter activity

HPAEpiC were co-transfected with NF-κB-Luc plasmid (Promega) and pRT-TK (Promega) with either miR-874-3p mimic or pcDNA-EGR3 and miR-874-3p mimic. Dual Luciferase Assay Kit was then used to detect the activities two days later.

RT-qPCR (reverse transcription quantitative real-time PCR)

Total RNAs were isolated from serum samples or HPAEpiC by Trizol (Invitrogen), and reverse-transcribed into cDNAs via PrimeScript RT Master Mix kit (Takara Biotechnology, Dalian, China). TB Green Premix Ex Taq (Takara, Dalian, China) was applied for qRT-PCR analysis. Caenorhabditis elegans miRNA (Cel-miR-39) was used as control to analyze miR-874-3p expression from serum samples. U6 was used as endogenous control to analyze miR-874-3p expression from cells. GAPDH was used as endogenous control to analyze TNF-α, IL-1β and EGR3 expression. Threshold cycle (Ct) value was determined to reflect fluorescent signal of amplification plot, and the relative gene expression was normalized to the controls by the 2−ΔΔCT method. The primer sequences were shown as below:

| Primer       | Sequence (5′-3′) |
|--------------|-----------------|
| GAPDH F      | ACCACAGTCCATGCCATCACC |
| GAPDH R      | TCCACCACCTGTTGCTGTA |
| TNF-α F      | ACTGAACCTCGGGGTGATTG |
| TNF-α R      | GCTTGGTGTTTGGTACGAC |
| IL-1β F      | CACCTCTTTTCTCTCATCCTTG |
| IL-1β R      | GTCTGGCTGTTGCTTCCCTTGTA |
| miR-874-3p F | GCCCTGAGGAAAGAAGCTGAG |
| miR-874-3p R | TGGATCCTCAAGGGGCTTGAC |
| EGR3 F       | CGAGGACCAAAAGGCCGAAGCTC |
| EGR3 R       | GATCAAGGGCATCTAACGTAAC |
| Cel-miR-39 F | CAGAGTAGCTCACCGGGGTGTTAAATC |
| Cel-miR-39 R | CCAGTGCGAGGCCTGAGGATAT |
| U6 F         | CTGCCCTCGGGCAGCAC |
| U6 R         | AACGCTTCCAGAATTTCGT |

Western blot

Total proteins were collected from HPAEpiC by radioimmunoprecipitation assay buffer (Beyotime Institute of Biotechnology, Beijing, China). Proteins (30 µg) were separated via SDS-PAGE, then transferred
to PVDF membrane. Membranes were blocked and incubated with the following primary antibodies: anti-EGR3 (1:2000; Cell Signaling Technology, Danvers, MA, USA), anti-Bcl-2 (1:2000; Cell Signaling Technology), anti-Bax (1:2500; Cell Signaling Technology), anti-cleaved caspase-3 (1:2500; Cell Signaling Technology), p65 (1:3000; Cell Signaling Technology) and anti-β-actin (1:3000; Cell Signaling Technology). After incubation with second antibodies (1:5000; Cell Signaling Technology) for 1 hour, the membranes were conducted with ECL chemiluminescence detection kit (Thermo Fisher), and the blots were visualized and quantified via Quantity One 4.6.2 software (Bio-Rad, Hercules, CA, USA).

Statistical analysis

Data were expressed as mean ± standard deviation. Statistical analyses between different groups were analyzed by GraphPad Prism 5.0 and determined by one-way analysis of variance and Student’s t-test. *p<0.05 was considered as statistically significant.

RESULTS

Reduced miR-874-3p in serum of pediatric patients with pneumonia

To unravel potential role of miR-874-3p in pediatric pneumonia, serum samples were collected from patients with pneumonia. Data from qRT-PCR demonstrated that miR-874-3p was reduced in serum of pediatric patients with pneumonia compared to the healthy control (Fig. 1A). The relationship between miR-874-3p expression and clinical characteristics of sick children was shown in Table 1. Symptom and signs, including cough, expectoration, dyspnea, and diarrhea, as well as laboratory tests, including white blood cells, neutrophils, and lymphocytes, were significantly associated with miR-874-3p expression (Table 1). HPAEpiC with LPS treatment was used to establish in vitro cell model of pneumonia. A dose-dependent reduction of miR-874-3p was identified in LPS-treated HPAEpiC (Fig. 1B), suggesting a potential role of miR-874-3p in pediatric pneumonia.

Table 1. Association between miR-874-3p expression and clinico-pathological parameters.

| Characteristics       | Number of patients | mir-874-3p | mir-874-3p | P value |
|-----------------------|--------------------|------------|------------|---------|
|                       |                    | Low expression (≤ medin) | High expression (> medin) | |
| Number                | 46                 | 27          | 19         | 0.446   |
| Gender                |                    | 0.806       |            |         |
| Male                  | 26                 | 14          | 12         |         |
| Female                | 20                 | 13          | 7          |         |
| Ages (years)          |                    | 0.005       |            |         |
| <10                   | 30                 | 18          | 12         |         |
| ≥10                   | 16                 | 9           | 7          |         |
| Symptom and signs     |                    | 0.001       |            |         |
| Cough                 | 29                 | 25 (92.59%) | 4 (21.05%) | p<0.001 |
| Expectoration         | 19                 | 17 (62.96%) | 2 (10.53%) | p<0.001 |
| Dyspnea               | 10                 | 9 (33.33%)  | 1 (5.26)   | 0.023   |
| Diarrhea              | 12                 | 10 (37.04%) | 2 (10.53%) | 0.044   |
| Laboratory tests      |                    | 0.002       |            |         |
| White blood cell (×10^9/L) | 11.9±1.4       | 10.27±1.97  |            |         |
| Neutrophils (%)       | 53.2±12.17        | 38.9±10.23  | p<0.001   |
| Lymphocyte (%)        | 43.8±17.12        | 59.12±18.23 | 0.005     |
MiR-874-3p attenuated LPS-induced inflammation in HPAEpiC

HPAEpiC was transfected with miR-874-3p mimic or inhibitor, and then incubated with 10 mg/L LPS for the evaluation of the role of miR-874-3p in pneumonia. LPS stimulated inflammation in HPAEpiC with increased mRNA (Fig. 2A) and protein (Fig. 2B) expression of TNF-α and IL-1β. However, transfection with miR-874-3p in LPS-treated HPAEpiC decreased TNF-α and IL-1β (Fig. 2A and 2B). Knockdown of miR-874-3p aggravated LPS-stimulated inflammation in HPAEpiC (Fig. 2A and 2B). **,##,&& p < 0.01.

Figure 2. MiR-874-3p attenuated LPS-induced inflammation in HPAEpiC
(A) Transfection with miR-874-3p mimic decreased mRNA expression of TNF-α and IL-1β in LPS-treated HPAEpiC, while miR-874-3p inhibitor transfection increased the expression. (B) Transfection with miR-874-3p mimic decreased protein expression of TNF-α and IL-1β in LPS-treated HPAEpiC, while miR-874-3p inhibitor transfection increased the expression. **,##,&& p < 0.01.

Figure 3. MiR-874-3p attenuated LPS-induced apoptosis in HPAEpiC
(A) Transfection with miR-874-3p mimic increased viability of LPS-treated HPAEpiC, while miR-874-3p inhibitor transfection decreased the cell viability. (B) Transfection with miR-874-3p mimic decreased apoptosis of LPS-treated HPAEpiC, while miR-874-3p inhibitor transfection increased apoptosis. (C) The apoptosis rate affected by miR-874-3p in LPS-treated HPAEpiC. (D) Transfection with miR-874-3p mimic increased Bcl-2 expression while decreased Bax and cleaved caspase-3 in LPS-treated HPAEpiC, while miR-874-3p inhibitor transfection reversed the effects. & p < 0.05, **,##,&& p < 0.01.
MIr-874-3p attenuated LPS-induced apoptosis in HPAEpiC

LPS decreased viability of HPAEpiC compared to the control (Fig. 3A). Transfection with miR-874-3p in LPS-treated HPAEpiC increased viability compared to NC mimic (Fig. 3A), while transfection with miR-874-3p inhibitor decreased viability compared to NC inhibitor (Fig. 3A). Similarly, LPS stimulated apoptosis of HPAEpiC (Fig. 3B and 3C) with reduction of Bcl-2 and increase of Bax and cleaved caspase-3 (Fig. 3D) compared to the control. However, transfection with miR-874-3p mimic in LPS-treated HPAEpiC suppressed apoptosis (Fig. 3B and 3C) with increase of Bcl-2 and reduction of Bax and cleaved caspase-3 (Fig. 3D). Knockdown of miR-874-3p aggravated LPS-stimulated apoptosis in HPAEpiC (Fig. 3B and 3C). Protein expression of Bcl-2 was down-regulated, while Bax and cleaved caspase-3 were up-regulated in LPS-treated HPAEpiC transfected with miR-874-3p inhibitor (Fig. 3D), suggesting that miR-874-3p could attenuate LPS-induced apoptosis in HPAEpiC.

Binding between miR-874-3p and EGR3

TargetScan analysis (http://www.targetscan.org/vert_71/) was used to identify the downstream target of miR-874-3p and EGR3 was predicted to bind with miR-874-3p (Fig. 4A). Over-expression of miR-874-3p decreased luciferase activity of EGR3-WT (Fig. 4B), while knockdown of miR-874-3p increased the activity (Fig. 4B). However, mutation at the binding site between miR-874-3p and EGR3 blocked the effect of miR-874-3p on luciferase activity of EGR3-MUT (Fig. 4B), suggesting that miR-874-3p could directly bind to EGR3. mRNA (Fig. 4C) and protein (Fig. 4D) expressions of EGR3 were reduced by miR-874-3p mimic, while knockdown of miR-874-3p increased EGR3 expression (Fig. 4C and 4D). EGR3 was dose-dependently increased in LPS-treated HPAEpiC (Fig. 4E), suggesting negative correlation with miR-874-3p and potential role of miR-874-3p/EGR3 in pediatric pneumonia.

EGR3 over-expression reversed the suppressive effects of miR-874-3p on inflammation and apoptosis in LPS-induced HPAEpiC

To investigate the role of miR-874-3p/EGR3 in pediatric pneumonia, HPAEpiC were co-transfected with pcDNA-EGR3 and miR-874-3p mimic, and then incubated with 10 mg/mL LPS. miR-874-3p over-expression reduced LPS-induced EGR3 expression (Fig. 5A), while co-transfection with miR-874-3p mimic and pcDNA-EGR3 reversed the suppressive effect of miR-874-3p on EGR3 expression (Fig. 5A). EGR3 over-expression aggravated LPS-induced increase of TNF-α and IL-1β (Fig. 5B), decrease of cell viability (Fig. 5C) and increase of apoptosis (Fig. 5D). However, over-expression of miR-
EGR3 over-expression counteracted the suppressive effects of miR-874-3p on inflammation and cell apoptosis in LPS-treated HPAEpiC. Over-expression of miR-874-3p decreased LPS-induced EGR3 expression, while co-transfection with miR-874-3p mimic and pcDNA-EGR3 reversed the suppressive effect of miR-874-3p on EGR3 expression. EGR3 over-expression aggravated LPS-induced decrease of cell viability, over-expression of miR-874-3p attenuated EGR3-induced decrease of cell viability. EGR3 over-expression aggravated LPS-induced increase of cell apoptosis, over-expression of miR-874-3p attenuated EGR3-induced increase of cell apoptosis. EGR3-induced decrease of Bcl-2, increase of Bax, cleaved caspase-3 and p65 were reversed by miR-874-3p mimic. EGR3-induced increase of NF-κB promoter activity was reversed by miR-874-3p mimic. $p<0.05$, $**p<0.01$.

**DISCUSSION**

Alveolar epithelial cells are the prime sites of bacterial or viral infection, and the infection could result in apoptosis and cytotoxicity of the epithelial cells (Herold et al., 2008). Prevention of apoptosis and cytotoxicity of alveolar epithelial cells could help ameliorate pneumonia (Kosmider et al., 2012). MiRNAs, with regulatory ability in cellular senescence, have been shown to be implicated in apoptosis of alveolar epithelial cells (Adameova et al., 2018). Since miR-874 has been reported to suppress cell proliferation and promote non-small cell lung cancer cell apoptosis (Wang et al., 2020), the potential regulatory roles of miR-874-3p in cytotoxicity of alveolar epithelial cells and development of pneumonia were evaluated in this study.
MiR-874-3p was down-regulated in serum of patients with pneumonia and LPS-treated HPAEpiC. LPS, as an endotoxin, could stimulate apoptosis and cytotoxicity. Therefore, LPS was widely used as a stimulatory model of acute pneumonia (Zhang et al., 2020). Our results indicated that LPS treatment reduced viability of HPAEpiC and enhanced apoptosis. Moreover, LPS could stimulate inflammatory response and promote pathogenesis of pneumonia (Meng et al., 2012). Proinflammatory factors TNF-α and IL-1β were found to be up-regulated in HPAEpiC post LPS treatment during this study. Alleviation of LPS-induced cytotoxicity and inflammation could help improve acute pneumonia (Zhang et al., 2019). We therefore validated the role of miR-874-3p in LPS-induced inflammation and apoptosis of HPAEpiC.

In line with previous study that miR-874 suppressed cell proliferation and promoted non-small cell lung cancer cell apoptosis (Wang et al., 2020), knockdown of miR-874-3p aggravated LPS-induced decrease of cell viability and increase of apoptosis in HPAEpiC. Moreover, LPS-induced secretion of TNF-α and IL-1β was also repressed by miR-874-3p over-expression, consistent with previous study that miR-874 could attenuate inflammatory response with decreased IL-1β and TNF-α in diabetic nephropathy (Yao et al., 2019). These results suggested that miR-874-3p could alleviate LPS-induced inflammation and apoptosis in alveolar epithelial cells, thus ameliorating development of pediatric pneumonia. Animal model of pneumonia should be established to further investigate the role of miR-874-3p in pediatric pneumonia.

MiR-874 could regulate necrosis through caspase-8 (Wang et al., 2013), and attenuate inflammatory response by toll-like receptor-4 (Yao et al., 2019). Our data demonstrated that miR-874-3p bind to EGR3. EGR3 was reported to promote inflammatory response in prostate cancer by increasing IL-6 and IL-8 (Baron et al., 2015). Our results showed that EGR3, increased in HPAEpiC after LPS treatment, counteracted the suppressive effects of miR-874-3p on inflammation and apoptosis in LPS-treated HPAEpiC. Therefore, miR-874-3p repressed LPS-induced apoptosis and inflammation in HPAEpiC via negative regulation of EGR3. EGR1 was found to be a neuronal target of NF-κB during Alzheimer’s disease (Snow & Albers, 2016). EGR3 could interact with NF-κB, p50, and p65 (Wieland et al., 2005), and promote activation of NF-κB pathway (Kurosaka et al., 2017). Our data revealed that over-expression of miR-874-3p reduced p65 expression and NF-κB promoter activity in LPS-treated HPAEpiC, while EGR3 over-expression enhanced p65 expression and NF-κB promoter activity and reversed the suppressive effects of miR-874-3p on NF-κB pathway. Activation of NF-κB could contribute to development of pneumonia (Jones et al., 2006), inhibition of NF-κB pathway could protect against LPS-induced apoptosis and inflammation of alveolar epithelial cells (Shao et al., 2017; Cheng et al., 2020), and attenuate pediatric pneumonia (Liu et al., 2017; Li et al., 2019). Therefore, the suppressive effect of miR-874-3p in LPS-induced inflammation and apoptosis of HPAEpiC might be dependent on inactivation of EGR3/NF-κB pathway.

CONCLUSION

MiR-874-3p was reduced in serum of pediatric patients with pneumonia. We found that miR-874-3p could promote viability of LPS-treated alveolar epithelial cells, while suppressing apoptosis and inflammation through targeting the EGR3/NF-κB pathway. This study provided a potential therapeutic target for pediatric pneumonia.

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Competing interests
The authors state that there are no conflicts of interest to disclose.

Ethics approval
This study was approved by the Ethics Committee of Zhejiang Zhoushan Putuo District Hospital of Traditional Chinese Medicine.

Statement of Informed Consent
Written informed consent was obtained from a legally authorized representative(s) for anonymized patient information to be published in this article.

Availability of data and materials
All data generated or analyzed during this study are included in this published article.

Authors’ contributions
Huirun Yang and Yang Dong designed the study, supervised data collection, Yan Zhou analyzed the data, interpreted the data, Huajun Li prepare the manuscript for publication and reviewed the draft of the manuscript. All authors have read and approved the manuscript.

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