Original Paper

MicroRNA-194 Regulates Lipopolysaccharide-Induced Cell Viability by Inactivation of Nuclear Factor- κ B Pathway

Fei Xie^a Lei Yang^b Lili Han^c Bin Yue^a

^aThe First Department of Pediatric, Cangzhou Central Hospital, Cangzhou, ^bThe Third Department of Orthopedics, Cangzhou Central Hospital, Cangzhou, ^cThe First Department of Neurology, Cangzhou Central Hospital, Cangzhou, China

Key Words

MicroRNA-194 • Lipopolysaccharide • Infantile pneumonia

Abstract:

The present study explored the functional role of microRNA (miR)-194 in lipopolysaccharide (LPS) induced lung cell injury, along with the underlying mechanisms and to reveal the potential role in infantile pneumonia. Human fibroblasts WI38 cells were transfected with miR-194 mimic or miR-194 inhibitor, and the transfection efficiency was confirmed by quantitative real-time polymerase chain reaction (qRT-PCR). Thereafter, the cells were treated with or without LPS, and then cell viability, cell apoptosis and mRNA and protein expressions of key proteins of nuclear factor kappa B (NF- κ B) pathway including inhibitor of NF- κ B (I κ B) α , p-65, and B-cell CLL/lymphoma (Bcl) 3 were analyzed. Results showed that overexpression and suppression of miR-194 was effective. Administration of LPS significantly decreased the cell viability and statistically promoted the percentages of apoptotic cells and increased the mRNA and protein expressions of p-65 and Bcl-3 but downregulated I κ B α compared to the control group ($P < 0.05$ or $P < 0.01$). LPS in combination with miR-194 suppression further enhanced the effects of LPS on cell viability and cell apoptosis compared to the LPS group ($P < 0.05$). In contrast, LPS in combination with miR-194 overexpression observably reversed the effects of LPS on cell viability, cell apoptosis and mRNA and protein expressions of the key proteins ($P < 0.05$ or $P < 0.01$). In conclusion, miR-194 increases the LPS-induced the inhibition of cell viability and increasing of the cell apoptosis by inhibition of NF- κ B pathway in WI38 cells. MiR-194 might be a potential targeted therapy for treatment of infantile pneumonia.

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Introduction

Pneumonia carries a high risk of morbidity and mortality, especially among children, causing about 23% of mortality in infants [1, 2]. Infantile pneumonia has been ranked as

Xie F

The First Department of Pediatric, Cangzhou Central Hospital
No.16, Xinhua West Road, Cangzhou, (China)
E-Mail xiefei00782@126.com

one of the three most important forms of pneumonia by the World Health Organization (WHO), and it has been classified as one of four critical diseases in child health in China [3]. Approximately 4, 000, 000 children die each year due to pneumonia among < 5 years old worldwide, particularly in developing countries (300, 000 new deaths are observed in China every year) [3]. Bacterial infection, viral infection, and *Mycoplasma pneumoniae* infection are the three main causes of infantile pneumonia. Although significant advances have been made in recent years in the diagnosis and treatment of infantile pneumonia, this disease still threatens the health of many children. Thus, there is an urgent need to explore new potential targeted therapies for treatment of infantile pneumonia.

MicroRNAs (miRNAs) are small short endogenous RNAs that regulate the expression of complementary messenger RNAs (mRNAs) at the post-transcriptional level by mainly binding to the 3'-untranslated region (UTR) of target mRNAs [4, 5]. It has been well known that miRNAs play an important role in regulating various cell functions such as cell proliferation, differentiation, migration, invasion, and apoptosis [6]. A growing number of studies have confirmed that miRNAs are differentially expressed in pneumonia, and many of them have been considered as biomarkers of pneumonia [7-9]. Among the miRNAs, miR-194 has been widely studied in various cancer cells, and shows complicated functions in regulating cell differentiation [10], cell migration [11], cell proliferation, apoptosis [12], and invasion [13]. For example, Dong et al. suggested that miR-194 inhibited epithelial to mesenchymal transition (EMT) of endometrial cancer (EC) cells by targeting oncogene B-cell-specific Moloney murine leukemia virus insertion site 1 (BMI-1) and inhibition of BMI-1 by miR-194 may have therapeutic potential in suppressing EC metastasis [14]. In addition, a previous study showed that miR-194 may serve as a valuable prognostic marker and promising interventional therapeutic target for hepatocellular carcinoma (HCC) [15]. Moreover, it has been reported that miR-194 plays a significant role in chondrogenic differentiation of human Adipose-derived stem cells (ASCs), which may provide a novel insight into cell manipulation methods during cartilage regeneration [16]. However, little information is available about the role of miR-194 in regulating lung cell injury, as well as its potential functional role in infantile pneumonia.

Therefore, in the present study, we aimed to explore the functional role of miR-194 in lung cell injury and potential role in infantile pneumonia. Human lung fibroblast WI38 cells were transfected with miR-194 mimic or inhibitor, and then the cells were stimulated with lipopolysaccharide (LPS) to induce lung cell injury and inflammatory response. Thereafter, the effects of miR-194 aberrant expression on cell viability and cell apoptosis were investigated. Moreover, we explored the involved potential signalling pathway. Our study might provide new insights into potential targeted therapies for treatment of infantile pneumonia.

Materials and Methods

Cell culture

Normal human fibroblasts WI38 cell line was obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA). The cells were cultured in Dulbecco's modified Eagle's medium (DMEM; Sigma-Aldrich, St Louis, USA) supplemented with 10% fetal bovine serum (FBS; Gibco, Grand Island, NY, USA), 100 units antibiotic/antimycotic solution (Invitrogen, Carlsbad, CA, USA) and 50 µg/mL gentamycin (Invitrogen) with 1 mM Na pyruvate at 37°C in a humidified atmosphere with 5% CO₂.

Cell treatment and plasmid transfection

Mature miR-194 mimic and miR-194 inhibitor were designed and synthesized by GenePharma (Shanghai, China). Cells without any vector transfection were regarded as the control group. Briefly, the cells (1 × 10⁵ cells/per well) were seeded on 96-well plates for 12 h and then were transiently transfected with 20 nM miR-194 mimic or miR-194 inhibitor following the manufacturer's instruction by Lipofectamine 2000 (Invitrogen) for 48 h. The detailed sequences were listed as following: miR-194 mimic sense 5'-UGUAAACAGCAAC UCCAUGUGGA-3' and mimic antisense 5'-CACAUUGGAGUUGCUGUUAACA-3'. miR-194

inhibitor 5'-UCCACAUGGAGUUGCUGUACA-3'. The transfection efficiency was confirmed by quantitative real-time polymerase chain reaction (qRT-PCR). The cells were then treated with or without 10 µg/ml *E.coli*-derived LPS (026:B6, Sigma-Aldrich) after plasmid transfection. The cell suspension was collected for further analyses.

qRT-PCR

Total RNA, including miRNA, was isolated from the transfected and treated cells by using miRNeasy Mini Kit (Qiagen, Valencia, CA, USA) according to the manufacturer's protocol. Reverse transcription was carried out using Multiscribe RT kit (Applied Biosystems, Foster City, CA, USA). The reverse transcription conditions were set at 30 min at 25°C, 30 min at 42°C, 5 min at 85°C, and a termination reaction at 4°C. The sequences of the primers were as follows: miR-194 forward primer: 5'-GCCCCGCTGTAACAGCAACTCCAT-3', reverse primer: 5'-GTGCAGGGTCCGAGGT-3'; U6 forward primer: 5'-CTCGCTTCGGC AGCACA-3', reverse primer: 5'-CTCGCTTCGGCAGCACA-3'; IκBα forward primer: 5'-CGGGTTCCTGCACTTGGCCATC-3', reverse primer: 5'-GTCCGGCCATTACAGGG CTC-3'; p65 forward primer: 5'-CTTAAGGAAAATTGAGGGAGGGCACGC-3', reverse primer: 5'-TGACTCAGTTTCCCTCCACACCGTCC-3'; Bcl-3 forward primer: 5'-CACGA AGGTTCCCTCTCTGGAAT-3', reverse primer: 5'-TGATCAATGTCTCCCAATCCC-3'; and GAPDH forward primer: 5'-CTCGCTTCGGCAGCACA-3', reverse primer: 5'-AAGCTTCACGAATTTGCGT-3'. All reactions were performed in triplicate and the data were analyzed by $2^{-\Delta\Delta C_T}$ method.

Cell viability

After incubation with LPS and/or transfection with miR-194 mimic or miR-194 inhibitor, the cell viability was analyzed by 3-(4, 5-dimethylthiazol-2-yl)-2,5-diphenyl-2Htetrazolium bromide (MTT) colorimetric assay according to a standardized method [17]. Briefly, the cells were seeded in 96-well plates and treated with LPS and/or transfected with miR-194 mimic or miR-194 inhibitor. After 1, 2, 3, and 4 days of treatment, 20 µL 5 mg/mL MTT (Invitrogen) was added to the plates and incubated at 37°C for 4 h. Thereafter, 100 µL dimethylsulfoxide (DMSO; Sigma-Aldrich) was added to dissolve the formazan crystals. Absorbance at 570 nm was read by a microplate reader (Bio-Rad Benchmark, Hercules, CA, USA). The data was graphed as relative cell viabilities [(OD570 drug)/ (OD570 virus control)] for each group.

Apoptosis assay

After incubation with LPS and/or transfection with miR-194 mimic or miR-194 inhibitor, the cell apoptosis was assessed by an Annexin V/ fluorescein isothiocyanate (FITC) and propidium iodide (PI) apoptosis detection kit (BD Biosciences, Heidelberg, Germany) according to the manufacturer's criterions. Briefly, after treatment and transfection for 48 h, the cells were harvested and suspended in Annexin-binding buffer, and then were incubated with Annexin V-FITC and PI for 15 min in the dark at room temperature. The apoptotic percentages of cells were analyzed by flow cytometry using a FACScan cytometer (BD Biosciences, USA) and the data was analyzed with FlowJo software (Tree Star, Ashland, OR).

Western blot analysis

After incubation with LPS and/or transfection with miR-194 mimic, the cell suspension was collected, centrifuged, and lysed in a RIPA lysis buffer containing protease inhibitor. The proteins were quantified using the BCA™ Protein Assay Kit (Pierce, Rockford, IL). The protein samples were then subjected to a 10-12% sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) gels and transferred to polyvinylidene fluoride (PVDF) membranes. After washing with phosphate buffer saline (PBS), the membranes were then blocked with 5% skim milk in Tris Buffered Saline with Tween (TBST) for 1 h. Subsequently, the membranes were incubated with the following primary antibodies overnight at 4°C: anti-IκBα antibody (rabbit mAb #4812; Cell Signaling Technology, Beverly, MA, USA); anti-phospho-IκBα (p-IκBα) antibody (rabbit mAb #5209; Cell Signaling Technology); anti-p65 antibody (rabbit mAb #8242; Cell Signaling Technology); anti-phospho-p65 (p-p65) antibody (rabbit mAb #3033; Cell Signaling Technology); and anti-B-cell CLL/lymphoma 3 (Bcl-3) antibody (rabbit mAb ab137499, Abcam, Cambridge, UK). The membranes were then washed three times with TBST and incubated with horseradish peroxidase (HRP)-conjugated secondary antibodies (Abcam) for 2 h. WEST-ZOL-plus Western Blot Detection System (Intron Biotechnology, Inc., Korea) was used to detect the protein bands. Probing for GAPDH (Abcam) was used as a loading control.

Statistical analysis

Each experiment was run in triplicate. The data was expressed as mean $\pm$ standard deviation (SD). All statistical analyses were performed with Statistic Package for Social Science (SPSS, version 19.0, SPSS Inc., Chicago, Illinois, USA) statistical software. One-way analysis of variance (ANOVA) was performed to calculate the P-values. A P-value of <0.05 was considered statistically significant.

Results

Transfection efficiency of miR-194

qRT-PCR was performed to confirm the transfection efficiency. As shown in Fig. 1A, the expression levels of miR-194 were significantly increased by transfection with miR-194 mimic compared to the control group ($P < 0.01$), and were statistically decreased by transfection with miR-194 inhibitor ($P < 0.05$). The results demonstrated that the transfection efficiency was apparent and could be used for further analysis.

Effects of miR-194 aberrant expression on cell viability

After abnormal expression of miR-194, we analyzed the effects of miR-194 aberrant expression on cell viability. The cell viability was determined by MTT. As shown in Fig. 1B, the results revealed that the cell viability was greatly lowered by LPS compared to the control group after 3 and 4 days of treatment (both $P < 0.01$). No significant differences were found in the cell viability after 1 and 2 days of treatment. In addition, we observed that LPS combined with overexpression of miR-194 prominently elevated the cell viability compared to the LPS group after 3 and 4 days of treatment ($P < 0.05$ or $P < 0.01$), and while suppression of miR-194 remarkably downregulated the cell viability compared to the LPS group after 3 and 4 days of treatment (both $P < 0.05$). The results indicated that overexpression of miR-194 could significantly promote the cell viability.

Effects of miR-194 aberrant expression on cell apoptosis

Moreover, the effects of miR-194 aberrant expression on the cell apoptosis were analyzed by flow cytometry. As shown in Fig. 2A and 2B, we found that compared to the control group, the percentages of apoptotic cells were remarkably promoted by LPS ($P < 0.05$). However, the percentages of apoptotic cells were strikingly downregulated by overexpression of miR-194

Fig. 1. (A) Transfection efficiency of miR-194. Human fibroblasts WI38 cells were transfected with miR-194 mimic or miR-194 inhibitor, and the transfection efficiency was confirmed by qRT-PCR. The results indicated high

transfection efficiency. (B) Effects of miR-194 aberrant expression on cell viability. The effects of miR-194 aberrant expression on cell viability were analyzed by MTT. The results showed that overexpression of miR-194 could dramatically increase the cell viability that was lowered by LPS at 3 and 4 days. Suppression of miR-194 further decreased the cell viability induced by LPS. MiR, microRNA; MTT, 3-(4, 5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide; LPS, lipopolysaccharide. ** $P < 0.01$ compared to the control group; # $P < 0.05$ compared to the LPS group; ## $P < 0.01$ compared to the LPS group.

Fig. 2. Effects of miR-194 aberrant expression on cell apoptosis. The effects of miR-194 aberrant expression on cell apoptosis were determined by flow cytometry. The Figure 3 (A) and (B) showed that overexpression of miR-194 could statistically decrease the cell apoptosis that was upregulated by LPS. Suppression of miR-194 further elevated the cell apoptosis induced by LPS. MiR, microRNA; LPS, lipopolysaccharide. * $P < 0.05$ compared to the control group; # $P < 0.05$ compared to the LPS group.

Fig. 3. Effects of miR-194 overexpression on the NF- κ B pathway key proteins expression. The effects of miR-194 overexpression on the NF- κ B pathway key proteins expression were measured by qRT-PCR and Western blot. (A) mRNA expressions of p/t-I κ B α , p/t-p65, and Bcl-3; (B), protein expressions of p/t-I κ B α , p/t-p65, and Bcl-3. MiR, microRNA; LPS, lipopolysaccharide; NF- κ B, nuclear factor kappa B; I κ B, inhibitor of NF- κ B; Bcl, B-cell CLL/lymphoma; qRT-PCR, quantitative real time polymerase chain reaction. * $P < 0.05$ compared to the control group; # $P < 0.05$ compared to the LPS group; ## $P < 0.01$ compared to the LPS group.

compared to the LPS group ($P < 0.05$), and while were distinctly increased by suppression of miR-194 ($P < 0.05$). These data suggested that overexpression of miR-194 could statistically inhibit the cell apoptosis.

Effects of miR-194 overexpression on the NF- κ B pathway key proteins expression

To further understand the underlying mechanism, both mRNA and protein expressions of NF- κ B pathway key proteins, I κ B α , p-I κ B α , p-65, p-p65, and Bcl-3, were measured after miR-194 aberrant expression and/or treatment with LPS. Non-treated cells were considered as the control group. As indicated in Fig. 3A, the data showed that the mRNA expression levels of p/t-p65 and Bcl-3 were all significantly raised but the levels of p/t-I κ B α were lowered by LPS compared to the control group ($P < 0.05$). Moreover, LPS combined with overexpression of miR-194 statistically reversed the effects of LPS ($P < 0.05$), while on the other hand, miR-194 overexpression alone further statistically increased the mRNA levels of p/t-I κ B α but decreased levels of p/t-p65 and Bcl-3 compared to the LPS group ($P < 0.01$). Similar results were observed in the protein levels (Fig. 3B). Our results indicated that overexpression of miR-194 could inhibit the NF- κ B pathway.

Discussion

In the present study, the results showed that LPS significantly downregulated the cell viability, upregulated the percentages of apoptotic cells, increased the mRNA and protein expressions of p-65 and Bcl-3 but decreased expression of I κ B α . Overexpression of miR-194 significantly attenuated the effects of LPS on cell viability, cell apoptosis, and the mRNA and protein expressions of I κ B α , p-65, and Bcl-3. However, suppression of miR-194 further enhanced the effects of LPS on cell viability and cell apoptosis. Our study indicated that miR-194 might be a protective factor in LPS-induced lung injury and overexpression of miR-194 might be a potential targeted therapy for treatment of infantile pneumonia.

The functional role of miR-194 has been studied for a long time; however, most studies have been focused on its biological functions in various tumour cells. Emerging studies suggest that miR-194 is considered as a tumour suppressor and its targets were identified in many cells and tissues. For example, Han et al. demonstrated that overexpression of miR-194 significantly repressed the proliferation, migration, and invasion of osteosarcoma cells *in vitro*, as well as tumour growth and pulmonary metastasis *in vivo* by targeting cadherin-2 (CDH2) and insulin-like growth factor 1 receptor (IGF-1R) [12]. In addition, it has been reported that miR-194 inhibited the EMT of EC cells [14] and gastric cancer cells [18]. MiR-194 has been identified as a prognostic marker of clear cell renal cell carcinoma (ccRCC) [19], myelodysplastic syndromes (MDS) [20], and HCC [15]. In addition to cancers, the biological functions of miR-194 in other diseases such as diabetes [21] and postmenopausal osteoporosis have also been investigated [22]. More recently, a study demonstrated that miR-194 was related with inflammatory response induced by palmitic acid (PA) [23]. In consideration of the above effects, we speculated that miR-194 might be involved in lung cell inflammatory response.

To confirm the hypothesis, the expression of miR-194 was first aberrantly expressed in human lung fibroblast WI38 cells by transfection with miR-194 mimic or inhibitor. After determining the high efficiency, we administrated LPS to the cells. LPS is a critical structural component of the outer membrane of Gram-negative bacteria, leading to systemic inflammatory response and nonspecific immune responses [24]. LPS is a widely used proinflammatory agent and is commonly accepted as a model of inducing pneumonia. The cell viability and the cell apoptosis were then measured after aberrant expression of miR-194 and administration of LPS. As expected, LPS significantly decreased the cell ability and increased the percentages of apoptotic cells compared to the control group, indicating a successful model for lung cell injury. Unlike the biological function of miR-194 in tumour cells, our study suggested that overexpression of miR-194 enhanced the cell viability and inhibited the cell apoptosis. But our study had similarities with those previous studies that miR-194 acted as a protective factor against diseases.

To further explore the underlying signalling pathway related to the effects of miR-194 aberrant expression on the cell viability and the cell apoptosis, we measured key proteins in

NF- κ B pathway including I κ B α , p-65, and Bcl-3. Activation of the NF- κ B signalling pathway plays a critical role in many different cell functions, such as inflammation, oncogenesis, cell apoptosis, and cell cycle progression [25]. Previous studies have demonstrated that LPS could induce the release of inflammatory cytokines and activate NF- κ B pathway [26-28]. I κ B α , a crucial negative regulator of NF- κ B activation, binds to NF- κ B, which blocks it in the cytoplasm and inhibits DNA binding [29]. Bcl-3, one of novel or atypical I κ B-like proteins, is also responsible for the activation of NF- κ B pathway. However, the functional role of Bcl-3 in activation of NF- κ B pathway is controversial. A few studies suggest that Bcl-3 participates in the removal of p50 and p52 homodimers from DNA and thus inhibits the transcriptional repressive effects of transcription activation domain (TAD) [30-32]. In contrast, other studies point that Bcl-3 interacts with p52 and p50, conferring upon them transcriptional activity [33-36]. p65, a subunit of NF- κ B, which is released from cytosolic I κ B α and targeted to the nucleus after phosphorylation and ubiquitination of I κ B α [37]. Our results revealed that LPS is capable of activating NF- κ B pathway by inhibiting the expression of I κ B α and upregulating the expression of p65 and Bcl-3. In contrast, overexpression of miR-194 reversed the effects of LPS on key proteins expressions, leading to inactivation of NF- κ B pathway.

Conclusion

Our results suggest that miR-194 functions as a protective factor in LPS induced lung cell injury by increasing lung cell viability and inhibiting cell apoptosis. These effects might be achieved by inhibition of NF- κ B pathway in WI38 cells. Additionally, miR-194 overexpression might be a potentially effective treatment of infantile pneumonia.

Abbreviations

LPS (lipopolysaccharide); miRNAs (microRNAs); CDH2 (cadherin-2); IGF-1R (insulin-like growth factor 1 receptor); EMT (epithelial-mesenchymal transition); MDS (myelodysplastic syndromes); HCC (hepatocellular carcinoma); PA (palmitic acid); TAD (transcription activation domain).

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FX, LY analysed the data and wrote the manuscript; FX, LY, LLH performed genotyping and analysed the data; BY performed computational analysis; All authors read and approved the final manuscript.

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Disclosure Statement

The authors declare that they have no competing interests.

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