Submitted: 05 March, 2021 Accepted: 12 April, 2021 Published: 08 July, 2021 DOI:10.22514/sv.2021.097

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ORIGINAL RESEARCH

miR-20a attenuates acute lung injury in septic rats via targeting TLR4

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Abstract

Background: Sepsis is most likely to cause lung damage in patients, and the detection rate and mortality rate are high. Here, we investigated the expression of miR-20a in sepsis-induced acute lung injury (ALI) rats and its effect on inflammatory response, and reveal its possible molecular mechanism.

Method: The model of acute lung injury caused by sepsis in rats was established by cecal ligation and puncture. The expression of miR-20a in lung tissue was determined by RT-qPCR. Acute lung injury rats were injected with 5 nmol miR-20a agomir or agomir NC every day for 3 days. Rats were sacrificed by arterial bleeding and lung tissues were removed. Serum interleukin (IL) -1β, IL-6, and tumor necrosis factor alpha (TNF-α) were detected by ELISA. HE staining was used to observe the pathology of lung tissue and calculate the pathological score of lung injury. Western blot to determine the level of TLR4 and nuclear transcription factor κB p65 (NF-κB p65) protein in lung tissue. The luciferase reporter assay was used to verify the binding effect of miR-20a on the 3 non-coding TLR4.

Results: We found that compared with that in Normal group, the expression of miR-20a in lung tissues of rats with ALI was decreased ($p < 0.05$). In miR-20a agomir group, the plasma level of IL-1β, IL-6, and TNF-α was significantly lower than that in agomir NC group and ALI group ($p < 0.05$), while higher than those in Normal group ($p < 0.05$). The HE staining results showed that the pathological score of lung injury in rats in miR-20a agomir group was lower than that of agomir NC group and ALI group ($p < 0.05$). Compared with agomir NC group and ALI group, the expression of TLR4 and NF-κB p65 in miR-20a agomir group was decreased ($p < 0.01$). The luciferase reporting experiment confirmed that TLR4 was a target gene of miR-20a.

Conclusion: To sum up, miR-20a exerts a protective effect on sepsis-induced ALI rats through its anti-inflammatory effect. The targeting of TLR4 by miR-20a may be an effective method to reduce the inflammatory response in sepsis-induced ALI.

Keywords

Sepsis; Acute lung injury; miR-20a; TLR4

1. Introduction

Sepsis is a systemic inflammatory response caused by infectious factors, which often leads to multiple organ dysfunction when it develops to a serious stage. As a most vulnerable target organ in sepsis, lung injury is the earliest and the highest incidence in patients with sepsis, and it is also one of the main causes of death in patients with sepsis [1]. Current studies have shown that inflammatory response is the key molecular mechanism of sepsis-induced ALI, and inhibition of inflammatory response in patients with sepsis plays a protective effect on ALI [2–4]. As an inflammation related miRNA, miR-20a plays a negatively regulatory role in the immune process of the body [5]. However, little study regarding whether miR-20a is involved in sepsis-induced ALI. This study aims to investigate the expression of miR-20a in sepsis-induced ALI and its effect on inflammatory response, and to clarify the underlying molecular mechanism.

2. Materials and methods

2.1 Main reagents

293T cells were from Yuchi Biotechnology Co., Ltd (Shanghai, China). Developer, protein double staining marker, Trizol reagent and one-step reverse transcription fluorescence quantitative kit were purchased from Shanghai Shenggong Biotechnology Co., Ltd (Shanghai, China). TLR4 (Abcam, Cambridge, MA, UK, ab13867). NF-κB p65 (Abcam, ab16502) and GAPDH (Abcam, ab9485) antibodies, HRP labeled second antibody (Abcam, ab6721), IL-1β (Abcam, ab216165)

ELISA reagent, IL-6 (Abcam, ab100712) ELISA reagent and TNF-α (ab208348) ELISA reagent were all purchased from aibokang Trading Co., Ltd (Shanghai, China). The dual luciferase detection and reporting system kit, miR-20a mimics, miR-20a agomir and their respective negative controls were purchased from Promega Corporation, Madison, USA.

2.2 ALI rats model

SPF-grade Sprague-Dawley (SD) rats (male, 4–5 weeks old, 80–100 g) were provided by the Laboratory Animal Science and Technology Center of Jiangxi University of Traditional Chinese Medicine (animal certificate number SCXK. Rats were raised in the Laboratory Animal Science Center of Nanchang University. ALI rats model was constructed as described by Rittirsch *et al*.'s [6] method. Briefly, in order to trigger a systemic inflammatory response, the cecal ligation and puncture model was conducted, then, bacteria were translocated into the blood compartment. The lung tissue in rats have obvious pathological damages such as destruction of alveolar wall integrity, congestion, edema, thickening, and inflammatory cell infiltration, which is considered as successful models of ALI. All animal experiments were approved by the Ethics Committee of the First Affiliated Hospital of Chengdu Medical College (Approval No 2019-013) [7].

2.3 Injection and grouping

Thirty successful rats model were randomly divided into three groups (10 rats in each group): (1) ALI group: pleural injection of PBS buffer; (2) Agomir NC group: pleural injection of Agomir NC (meaningless sequence); (3) miR-20a agomir group: miR-20a agomir was injected into the chest cavity. The rats were injected for three consecutive days, and the injection dose was referred to the literature [8]. On the 4th day, the rats were sacrificed by arterial bloodletting and lung tissue was removed. In addition, 10 rats of the same age were selected as normal control group.

2.4 RT-qPCR

The expression of miR-20a in different groups was detected by RT-qPCR. RNA was extracted from the lung tissues of rats by Trizol, and the expression of miR-20a was detected according to the instructions of the one-step reverse transcription fluorescence quantitative kit. miR-20a, forward primer 5′-CGGCGCTAAAGTGCTTATAGTGC-3′ and reverse primers 5′-ATCCAGTGCAGGGTCCGAGG-3′; U6, forward primer 5′-GCTTCGGCAGCACATATACTAAAAT-3, reverse primers 5′-CGCTTCACGAATTTGCGTGTCAT-3′.

2.5 ELISA

The expression levels of inflammatory cytokines (IL-1β, IL-6 and TNF-α) were detected by ELISA. The blood was centrifuged (3000 r/min, 5 min), and the plasma was collected and used for analysis. The procedure was carried out based on the instructions of ELISA detection kit, and the standard curve was drawn for determination.

2.6 HE staining

The lung tissues were collected (1.5 cm $\times$ 1.5 cm $\times$ 1.4 cm), fixed by 10% formaldehyde and stained by HE after paraffin embedding. The pathological score of lung injury was obtained as described previously [9]: (1) edema, (2) inflammatory cell infiltration, and (3) tissue hemorrhage. Each item has a minimum score of 0 and a maximum score of 4. Among them: no damage, count for 0; slight damage (about 25% bleeding range), count for 1; moderate damage (about 50% bleeding range), count for 2; severe damage (about 75% bleeding range), count for 3; the most serious damage (about the whole field of vision bleeding), count for 4. The total score of the degree of lung injury was the total score of each pathological score of lung injury.

2.7 Western blot

The protein level was detected by Western blot. The lung tissues of each group were added with RIPA lysate, lysed on ice (20 minutes), centrifuged (4 °C, 13000 rpm, 20 mins), and the supernatant was finally taken and the protein content was determined using the BCA kit. The protein solution (35 μg/30 μL) was used for SDS-PAGE, transferred to membrane and blocked with blocking solution (2% BSA). Subsequently, the primary antibody (with GAPDH antibody as reference) was added (4 °C, overnight). After that, the secondary antibody was added and incubated at room temperature for 1 h. After ECL exposure, the Alpha Imager HP image documentation system (Alpha Innotech, San Leandro, CA) was used for analyzing the results.

2.8 Luciferase reporter assay

Luciferase reporter assay was performed to investigate the binding sites between miR-20a and TLR4. The wild-type (WT) and mutant (MUT) TLR4 3'-UTR dual fluorescent reporter plasmids were firstly constructed. The 293T cells in logarithmic growth phase were seeded into 12-well cell plates (10^5 cells/well), and the TLR4-WT, TLR4-MUT and miR-20a mimics or mimics NC negative controls were co-transfected into 293T cells. Each experiment was replicated for 6 times. After 48 h of incubation, the luciferase activity was detected according to the dual luciferase reporter gene detection kit.

2.9 Statistics

All data were expressed as mean $\pm$ standard deviation (SD) and analyzed by using SPSS 21.0 software. With normal distribution, the independent sample t test was used for comparion between two groups, and the one-way ANOVA was used for comparison among multiple groups ($\alpha = 0.05$). p value less than 0.05 was considered statistically significant.

3. Results

3.1 Expression of miR-20a in the lung tissue from sepsis-induced ALI rats

RT-qPCR results (Fig. 1) showed that the relative expression of miR-20a (2.03 $\pm$ 0.06) in the lung tissue from sepsis-induced ALI rats was significantly lower than that from normal

rats (10.05 ± 0.49) (t = 16.29, $p < 0.001$). To explore the functional role of miR-20a in the progression of ALI, miR-20a was overexpressed in ALI rats. The expression of miR-20a in the lung tissue from rats in the miR-20a agomir group (5.03 ± 0.17) was significantly up-regulated as compared with that in the agomir NC group (2.02 ± 0.06) (t = 16.50, $p < 0.001$).

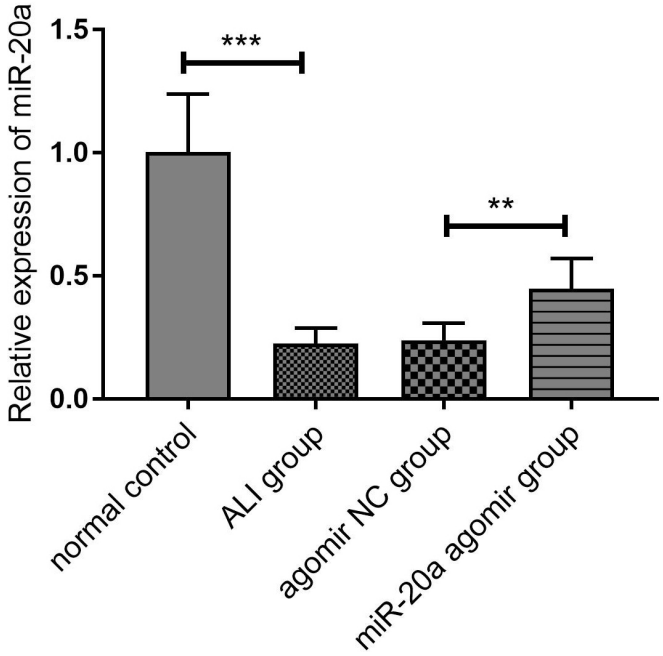

FIGURE 1. The expression of miR-20a in different groups. qRT-PCR was used to detect the expression of miR-20a in ALI, miR-20a agomir group and their controls. n = 6 in each group. Data were expressed as mean ± SD. ** $p < 0.01$ and *** $p < 0.001$ represent statistically difference.

3.2 Effects of miR-20a agomir on the expression of IL-1β, IL-6 and TNF-α in plasma from sepsis-induced ALI rats

The expression levels of IL-1β, IL-6 and TNF-α in plasma were significantly elevated in sepsis-induced ALI rats as compared with that in normal control ($p < 0.05$), suggesting an inflammatory response. However, the level of these cytokines was significantly reduced in the plasma from ALI rats after injection of miR-20a agomir compared with agomir NC group ($p < 0.05$), indicating that the expression of these factors can be suppressed by miR-20a. Detailed information was shown in Table 1.

3.3 Effect of miR-20a agomir on the histological changes from sepsis-induced ALI rats

HE staining results showed that the structure of alveoli and bronchus in the normal control group was clear and complete, and there was no inflammatory cell infiltration and exudate. In the lung tissue of ALI group and agomir NC group, the alveolar septum was thickening, the obvious dilatation of blood vessels was found in the interstitium, and a large number of inflammatory cells in the lung interstitium around the bronchus were infiltration, and the alveolar cavity was filled with serous exudate. In the miR-20a agomir group, the pathological injury of lung tissue was mild, and a small amount of inflammatory cell infiltration and exudate were observed (Fig. 2). The lung injury score in miR-20a agomir group, agomir NC group, ALI group and normal control was 3.40 ± 0.34, 9.60 ± 0.31, 9.70 ± 0.26 and 0.50 ± 0.22, respectively. Taken together, the pathological score of lung injury in miR-20a agomir group was lower than that in agomir NC group and ALI group ($p < 0.05$), but higher than that in normal control ($p < 0.05$), indicating the protective effects of miR-20a agomir on lung tissue.

3.4 Effect of miR-20a agomir on the expression of TLR4 and NF-κB p65 in lung tissue from sepsis-induced ALI rats

The protein expression levels of TLR4 and NF-κB p65 in lung tissue were significantly up-regulated in miR-20a agomir group compared with that in control group (Fig. 3, $p < 0.05$), which were significantly reduced as compared with that in the NC group and ALI group ($p < 0.05$). These results indicated that miR-20a agomir could induce the expression of TLR4 and NF-κB p65 in lung tissue from sepsis-induced ALI rats.

3.5 The direct relationship between miR-20a and TLR4

The binding site between miR-20a and TLR4 was predicted by Targetscan bioinformatics website. The sequence was shown in the left panel of Fig. 3, and the results of luciferase assay showed that in TLR4 wild type 3'-UTR, the relative activity of luciferase in miR-20a mimics group was significantly lower than that in mimics NC group ($p < 0.001$). However, in TLR4 mutant 3'-UTR, the relative activity of luciferase in miR-20a mimics group did not show any change as compared with mimics NC group (Fig. 4, right panel). These results proved that miR-20a could directly target the 3'-UTR of TLR4.

4. Discussion

As one of small RNAs, miRNA has been reported related to the occurrence and development of tumor [10], cardiovascular disease [11], diabetes [12], human genetic disease [13] and nervous system disease [14]. Wu Songlin *et al.* [15] showed that miR-125b was significantly decreased in lipopolysaccharide (LPS)-induced ALI rats, and was negatively correlated with the expression of TNF-α and IL-6. Leng *et al.* [15] found that miR-483-5p was up-regulated in the lung tissue of sepsis-induced ALI mice. By inhibiting the expression of protein inhibitor activating STAT1 (PIAS1), miR-483-5p could promote the inflammatory response and induce the apoptosis of pulmonary microvascular endothelial cells and thus aggravated ALI. Therefore, miRNA may play a key role in the occurrence and development of ALI.

miR-20a, one of the members of miR-17/92 cluster, is located on chromosome 13q31.1 and mainly participates in inflammatory response [5]. Li *et al.* [16] pointed out that overexpression of miR-20a may target the regulation of thioredoxin interacting protein (TXNIP) and reduce the activity of NLRP3. Chen *et al.* [17] have shown that miR-20a

TABLE 1. Effect of miR-20a agamir on the levels of IL-1β, IL-6 and TNF-α in plasma from sepsis-induced ALI rats.

Project	miR-20a agomir group	Agomir NC group	ALI group	Normal control
IL-1β (ng/L)	$85.65 \pm 0.63^{*\#\Delta}$	143.20 ± 0.52	144.20 ± 0.66	74.12 ± 0.90
IL-6 (μg/L)	$0.60 \pm 0.01^{*\#\Delta}$	0.87 ± 0.01	0.88 ± 0.01	0.50 ± 0.01
TNF-α (μg/L)	$0.30 \pm 0.01^{*\#\Delta}$	0.73 ± 0.01	0.72 ± 0.01	0.20 ± 0.01

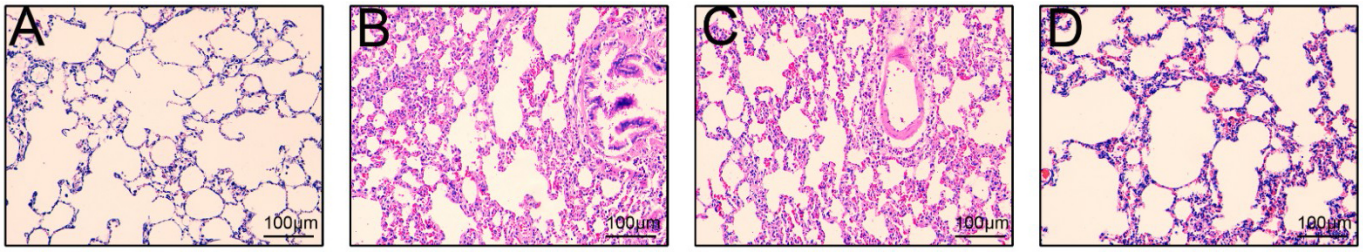

FIGURE 2. Effect of miR-20a on pathological morphology of lung tissue (HE $\times$ 200). The lung tissue of rats with sepsis was fixed with 10% formaldehyde, and the pathological changes were observed by HE staining after paraffin embedding. The lung tissue appearance of rats in normal control (A), ALI group (B), agomir NC group (C) and miR-20a agomir group (D). n = 6 in each group. Data were expressed as mean $\pm$ SD.

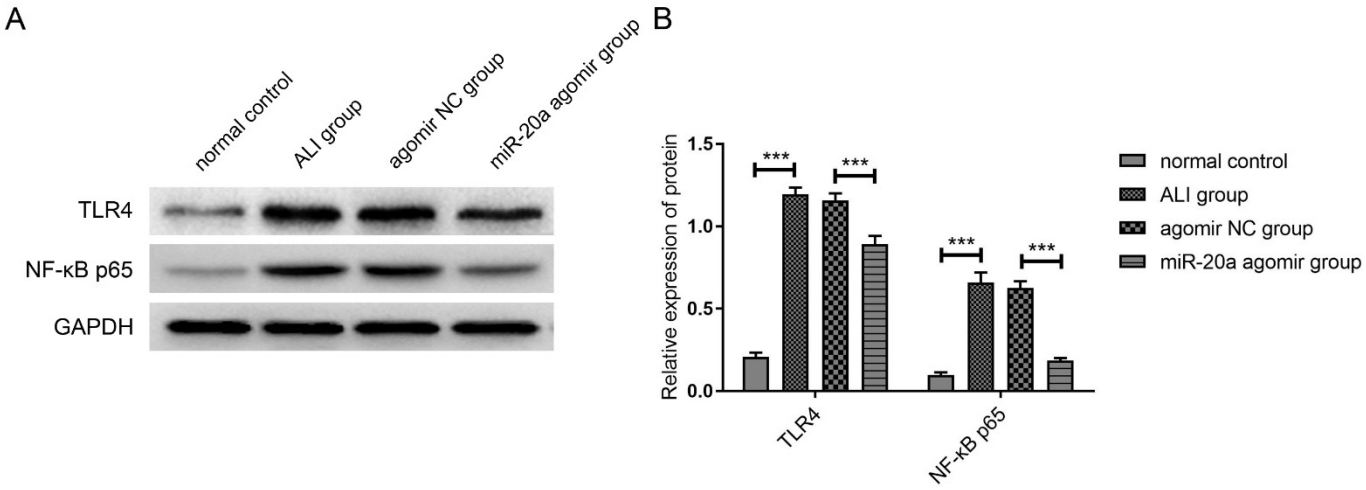

FIGURE 3. miR-20a inhibits the expression of TLR4 and NF-κB p65 in rat lung tissue. (A) The expressions of TLR4, NF-κB p65 and GAPDH in normal control, ALI group, agomir NC group and miR-20a agomir group were detected by Western blot. (B) Alpha Imager HP gel imaging system was used to analyze the relative protein expression levels of TLR4 and NF-κB p65 in lung tissues in four groups. n = 6 in each group. Data were expressed as mean $\pm$ SD. ** $p < 0.01$ and *** $p < 0.001$ represent statistically difference.

could reduce inflammation by regulating TLR4 and TXNIP signaling pathways, thereby reducing the damage of human aortic endothelial cells (HAECs) induced by oxidized low-density lipoprotein (ox-LDL). Zhu *et al.* [18] pointed out that miR-20a could regulate macrophage infiltration, phagocytosis and pro-inflammatory cytokine secretion by targeting signal regulated protein α 1 (SIRP α). However, little study regarding whether miR-20a is involved in sepsis-induced ALI. This study found that miR-20a expression in the lung tissue of sepsis-induced ALI rats was decreased. Therefore, we speculated that miR-20a may play a role in the pathological process of ALI. To further explore the role of miR-20a in ALI, miR-20a agomir was injected into sepsis-induced ALI rats, and the results showed that the levels of IL-1β, IL-6, and TNF-α in serum were significantly reduced, and the pathological score of lung injury was significantly decreased. It is further confirmed that miR-20a plays a protective role in sepsis-induced ALI rats through its anti-inflammatory effect.

Toll like receptor 4 (TLR4) is an innate immune receptor, which is involved in immune regulation and inflammatory response. The inflammatory response caused by the activation of TLR-4 signaling pathway is the key molecular mechanism of sepsis-induced ALI. In particular, TLR4, MyD88, NF-κB, TNF-α, IL-1β, IL-6 and other inflammatory mediators are the most studied molecules in TLR-4 signaling pathway, and are also considered as the targets of most drugs for the intervention of sepsis-induced ALI [19]. Liu Hui *et al.* [20] also showed that TLR4 inhibitor can suppress the inflammatory response mediated by NF-κB signaling pathway in septic rats and play a therapeutic role in the progression of

A

TLR4 wild-type 3'-UTR:5' gaCUGAACUGGGUGUU ***CACUUU*** u 3'

miR-20a : 3' gaugGACGUGAUUC ***GUGAAA*** u 5'

TLR4 mutant 3'-UTR : 5' gaCUGAACUGGGUGUU ***GUGAAA*** u 3'

B

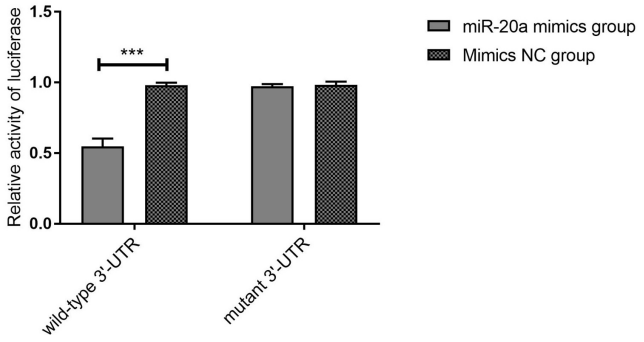

FIGURE 4. Targeting relationship between miR-20a and TLR4. (A) The TargetScan bioinformatics site was used to predict the binding sites between miR-20a and TLR4. (B) The relative activity values of luciferase were detected using the dual luciferase reporter assay kit. n = 6 in each group. Data were expressed as mean ± SD. *** $p < 0.001$ represent statistically difference.

ALI. Through bioinformatics website, it was predicted that miR-20a could complement the 3'-UTR region of TLR4. In this study, the dual luciferase assay was used to verify the targeted regulatory relationship between miR-20a and TLR4. In addition, miR-20a agomir reduced the expression of TLR4 and NF-κB p65, and reduced the inflammatory response in ALI rats. Taken together, it can be inferred that miR-20a can inhibit the inflammatory response in the progress of ALI by blocking the NF-κB pathway via targeting TLR4, which was similar to the results from Li [21]. Their results showed that miR-20a inhibited the production of inflammatory cytokines in LPS stimulated human gingival fibroblasts by activating the TLR4 signaling pathway.

This study demonstrated the effect of miR-20a on inflammatory response in sepsis-induced ALI was by inhibiting TLR4/NF-κB signaling pathway. Based on the fact that one miRNA may regulate several target genes, the regulatory mechanisms of miRNA on target genes are complex. Therefore, further analysis on revealing the specific mechanism of miR-20a in sepsis ALI was needed.

AUTHOR CONTRIBUTIONS

SPL and YS designed the study, supervised the data collection. XMT analyzed the data, interpreted the data. LW and XYC prepare the manuscript for publication and reviewed the draft of the manuscript. All authors have read and approved the manuscript.

ETHICS APPROVAL AND CONSENT TO PARTICIPATE

Ethical approval was obtained from the Ethics Committee of the First Affiliated Hospital of Chengdu Medical College (Approval No 2019-013).

ACKNOWLEDGMENT

Thanks to all the peer reviewers for their opinions and suggestions.

FUNDING

This research received no external funding.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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How to cite this article: Shuping Li, Yang Sun, Xingming Tang, Li Wang, Xiaoyuan Cheng. miR-20a attenuates acute lung injury in septic rats via targeting TLR4. Signa Vitae. 2021;17(4):157-162. doi:10.22514/sv.2021.097.