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# Long noncoding RNA HAGLROS regulates cell apoptosis and autophagy in lipopolysaccharides-induced WI-38 cells via modulating miR-100/NF-κB axis

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ABSTRACT

Pneumonia is a lower respiratory disease caused by pathogens or other factors. This study aimed to explore the roles and mechanism of long noncoding RNA HAGLROS in lipopolysaccharides (LPS)-induced inflammatory injury in pneumonia. The HAGLROS expression in serum of patients with acute stage pneumonia was detected. To induce pulmonary injury, WI-38 human lung fibroblasts were stimulated with lipopolysaccharides (LPS). The HAGLROS expressions in LPS-treated WI-38 cells and the effects of HAGLROS knockdown on the viability, apoptosis, and autophagy of LPS-induced cells were detected. Moreover, the regulatory relationship between HAGLROS and miR-100 was explored as well as the functional targets of miR-100 were identified. Furthermore, the regulatory relationship between miR-100 and PI3K/AKT/NF-κB pathway was elucidated. LncRNA HAGLROS was higher expressed in serum of patients with acute stage pneumonia compared with that in serum of healthy control. LPS caused WI-38 cell injury and increased HAGLROS levels. Downregulation of HAGLROS alleviated LPS-induced cell injury via increasing cell viability, and inhibiting apoptosis and autophagy. Moreover, there was a negative correlation between HAGLROS and miR-100, and the effects of HAGLROS downregulation on LPS-induced apoptosis and autophagy in WI-38 cells were by regulation of miR-100. Furthermore, NFκB3 was verified as a functional target of miR-100 and effects of miR-100 inhibition on LPS-induced WI-38 cell injury were alleviated by knockdown of NFκB3. Besides, Knockdown of HAGLROS inhibited the activation of PI3K/AKT/NF-κB pathway. Our findings reveal that downregulation of HAGLROS may alleviate LPS-induced inflammatory injury in WI-38 cells via modulating miR-100/NF-κB axis. HAGLROS/miR-100/NF-κB axis may provide a new strategy for treating acute stage of pneumonia.



## 1. Introduction

Pneumonia is a lower respiratory disease caused by pathogens or other factors. The typical symptoms of pneumonia are dry cough, chest pain, fever, shortness of breath and fixed wet lung sound [1,2]. Moreover, pneumonia represents a major cause of death, especially in children and elderly people [3,4]. Community-acquired pneumonia, a common type of pneumonia, is responsible for high rates of morbidity and mortality worldwide [5]. Therefore, it is urgent to elucidate the key molecular mechanism underlying pneumonia, thus improving the clinical outcomes of this disease.

Accumulating evidences have disclosed several molecular mechanisms underlying pneumonia. For instance, high expression levels of interleukin-10 and interferon-induced protein 10 are associated with more severe hypoxic pneumonia in human immunodeficiency virus (HIV)-infected infants [6]. Four risk single-nucleotide polymorphisms (SNPs) located in chromosomes 1 and 17 contribute to the development of severe pneumonia in A/H1N1 infection [7]. Moreover, RNA-sequencing analysis has identified several miRNAs (hsa-let-7f-1, hsa-miR-455 and hsa-miR-200b) that may play a role in pneumonia pathogenesis [8]. However, the association of long noncoding RNAs (lncRNAs) with pneumonia has not been fully investigated.

LncRNAs are transcribed RNAs with length more than 200

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nucleotides, which are not translated into proteins [9]. Growing evidences link the dysregulation of lncRNAs to the pathophysiological development of inflammatory diseases, such as inflammatory bowel disease [10], rheumatoid arthritis and osteoarthritis [11], as well as lung diseases, including pulmonary fibrosis [12] and lung cancer [13]. Although a recent lncRNA sequencing analysis has identified the potential Key Long Non-Coding lncRNAs associated with pneumonia [14], the crucial lncRNAs that are correlated with pneumonia development are largely unknown. HAGLROS is a 699 bp lncRNA that has recently been found to promote the malignant progression of gastric cancer cells [15]. Nevertheless, the key role of HAGLROS in pneumonia pathogenesis has not been reported.

In the present study, whether HAGLROS was dysregulated in serum of patients with acute stage pneumonia was detected. To induce pulmonary injury, WI-38 human lung fibroblasts were stimulated with lipopolysaccharides (LPS). The HAGLROS expressions in LPS-treated WI-38 cells and the effects of HAGLROS knockdown on the viability, apoptosis, and autophagy of LPS-induced cells were detected. Moreover, it has been reported that HAGLROS serves as a sponge for miR-100-5p in gastric cancer cells [15], thus the regulatory relationship between HAGLROS and miR-100 was explored as well as the functional targets of miR-100 were identified. Furthermore Qingfei Tongluo Ointment, a traditional Chinese medicine, is found to reduce the pathological injury of lung tissue in pneumonia rats via regulating the activation of PI3K/AKT/NF-κB pathway [16], we thus investigated the regulatory relationship between miR-100 and PI3K/AKT/NF-κB pathway. Our data will lay a theoretical basis for better understanding of the molecular mechanism underlying pneumonia and may provide a new insight for the treatment of this disease.

## 2. Materials and methods

### 2.1. Patients and blood collection

This study was approved by the ethics committee of our hospital. 20 patients (mean age, 21.6 ± 2.4 years; 16 males and 4 males) who were diagnosed as acute stage pneumonia were included. Patients experienced other complications or had received anti-inflammatory therapy were excluded. 20 healthy individuals (mean age, 23.6 ± 3.6 years; 15 males and 5 males) were also recruited as controls. A total of 3 mL of fasting peripheral venous blood was collected from all enrolled individuals. Followed by centrifugation under 2000 r/min, serum was collected and then stored in a 80 °C freezer. All enrolled individuals provided a signed informed consent for using their serum in research.

### 2.2. Cell culture

Normal human fibroblast cell line WI-38 (ATCC, Manassas, VA, USA) was 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), 50 μg/mL gentamycin (Invitrogen, Carlsbad, CA, USA) with 1 mM Na pyruvate, and 100 units antibiotic/antimycotic solution (Invitrogen), and then maintained in a humidified atmosphere with 5% $CO_2$ at 37 °C.

### 2.3. Transient transfection and treatment

The WI-38 cells ($2 \times 10^5$) were seeded in 6-well plates and incubated overnight. Cells were transfected with short-hairpin RNA against HAGLROS, 50 nM of miR-100 mimic, 150 nM of miR-100 inhibitor, siRNA against NFKB3 or their negative controls (NC) using Lipofectamine 2000 Reagent (Life Technologies Corporation, Grand Island, NY, USA). After plasmid transfection, 10 μg/ml *E.coli*-derived lipopolysaccharide (LPS) from strains 026:B6 (Sigma-Aldrich) were added to treat different transfected cells, and cells without LPS treatment were used as blank control. After 24, 48, 72 h of LPS treatment, cell suspension was collected for further analyses.

### 2.4. Real-time PCR

Total RNA was extracted using Trizol reagent (Invitrogen). Approximately 2 μg of total RNA was reverse-transcribed into cDNA using an M-MLV Reverse Transcriptase kit (Invitrogen). Relative gene expression was analyzed by real-time qRT-PCR using a standard SYBR Green PCR kit (Toyobo, Osaka, Japan). U6 and β-actin were used as references for miRNAs and mRNAs, respectively. The relative quantitation of gene expression was determined using $2^{-\Delta\Delta CT}$ method. Each sample was analyzed in triplicate.

### 2.5. Vector construction and dual-luciferase reporter assay

The 3′-UTR of NFKB3 containing miR-100 binding site (NFKB3-WT) was synthesized by Invitrogen (Shanghai, China) and then inserted downstream of the luciferase reporter in the pmirGLO Dual-Luciferase miRNA Target Expression Vector (Promega, Madison, USA). The identical flanking nucleotides of NFKB3 3′-UTR containing the mutated miR-100 target sequence (NFKB3-MUT) was used as the control. For dual-luciferase reporter assay, WI-38 cells were plated in 24-well plates and transfected with miR-100 mimic or mimic control and with the above constructed luciferase vectors. After 48 h transfection, cells were harvested for measuring the luciferase activity using the Dual-Glo luciferase assay kit (Promega).

### 2.6. Cell viability assay

MTT assay was applied for determining cell viability of different groups. Briefly, approximately $2 \times 10^3$ WI-38 cells were plated in a 96-well plate and incubated overnight. At various times following different treatment, the medium was removed, and 20 μL MTT (5 mg/mL, Sigma-Aldrich) was added to incubate cells for 4 h at 37 °C. The harvested formazan precipitates following centrifugation were then dissolved in 150 μL of dimethyl sulfoxide. The absorbance of each well at 470 nm was then detected using a MRX II absorbance reader (DYNEX Technologies, Chantilly, Virginia, USA).

### 2.7. Detection of cell apoptosis

Following different treatment, $1 \times 10^3$ cells were harvested, washed twice with pre-chilled PBS and resuspended in 100 μL binding buffer. According to the manufacturer's protocol of Annexin V-FITC Apoptosis Detection Kit (BD Biosciences, San Jose, CA, USA), double-staining of Annexin V and propidium iodide (PI) was then performed. Cell apoptosis was the detected by flow cytometry within 1 h by means of the BD LSRII Flow Cytometer System with FACSDiva Software.

### 2.8. Western blot

Different treated cells were collected and lysed with cell lysis buffer (Beyotime, Haimen, China) for extraction of total protein. Followed by determining the protein concentration by Bradford reagent (Biorad laboratories, CA, USA), protein extracts (30 μg per lane) were separated on 12% SDS-polyacrylamide gels and transferred onto polyvinylidene fluoride (PVDF) membranes (Millipore, Billerica, MA, USA). By incubation with primary antibodies to against LC3I, LC3II, Beclin1, NFKB3, p-NFKB3, p-PI3K, PI3K, AKT, p-AKT, Bcl-2, Bax, caspase-3, caspase-9, and β-actin (1:1000, Santa