Life Sciences 228 (2019) 189–197

Contents lists available at ScienceDirect

Life Sciences

journal homepage: www.elsevier.com/locate/lifescie

Review article

Long noncoding RNA SNHG16 targets miR-146a-5p/CCL5 to regulate LPS-induced WI-38 cell apoptosis and inflammation in acute pneumonia

Zhiming Zhou[a], Yuyin Zhu[a], Guosheng Gao[b], Yena Zhang[a,*]

[a] Department of Pulmonary Medicine, HwaMei Hospital, University of Chinese Academy of Sciences, 315010 Ningbo, Zhejiang Province, China
[b] Department of Laboratory, HwaMei Hospital, University of Chinese Academy of Sciences, 315010 Ningbo, Zhejiang Province, China

ARTICLE INFO

Keywords:
Pneumonia
SNHG16
miR-146a-5p
JNK and NF-κB pathways

ABSTRACT

Aims: Aberrant expression of the lncRNA small nucleolar RNA host gene 16 (SNHG16) has been researched in multiple cancers and inflammatory diseases. This study was intended to investigate the effect of SNHG16 in vitro model of pneumonia and explore the potential mechanism.

Main methods: The LPS-induced pulmonary injury model was established in WI-38 human lung fibroblasts cells. SNHG16 and miR-146a-5p expression levels were altered by transfection assay and were evaluated by qRT-PCR. Cell viability and apoptosis were respectively assessed by CCK-8 assay and flow cytometry analysis. The combination of miR-146a-5p and SNHG16 were demonstrated by luciferase reporter assay, RNA immunoprecipitation (RIP) assay and RNA pull-down assay. Associated inflammatory factors expression levels and productions were determined by qRT-PCR, western blotting and Enzyme-linked immunosorbent (ELISA) assay, respectively. Main proteins related apoptosis, c-Jun N-terminal kinase (JNK) pathway and nuclear factor (NF)-κB pathway were also analyzed by western blotting.

Key findings: SNHG16 was highly expressed in serum of acute stage pneumonia patients. SNHG16 was up-regulated in LPS-treated WI-38 cell model and SNHG16 knockdown obviously mitigated LPS-induced cell injury by promoting viability, restraining apoptosis and production of inflammatory cytokines. SNHG16 functioned as a competitive endogenous RNA (ceRNA) by efficaciously binding to miR-146a-5p and then restoring C—C motif chemokine ligand 5 (CCL5) expression. Besides, miR-146a-5p inhibitor abolished the function of SNHG16 knockdown on cell injury, JNK and NF-κB pathways.

Significance: SNHG16 regulated LPS-induced inflammation injury in WI-38 cells through competitively binding miR-146a-5p with CCL5 further mediating JNK and NF-κB pathways, which sheds novel light on diagnostics and therapeutics in pneumonia.

1. Introduction

Pneumonia is one of lower respiratory illnesses mainly caused by pathogens including bacteria, viruses and fungi, accompanied with typically clinical symptoms of fever, cough, shortness of breath, chest pain even respiratory failure and heart failure [1,2]. Pneumonia is a main infectious disease with the world's leading mortality and morbidity rates, particularly in children and seniors [3–5]. It is well known that pneumonia is associated with inflammatory stimulation from microorganisms, for instance, endotoxin, which is one of the leading causes of severe pneumonia [6]. As a potent endotoxin, lipopolysaccharide (LPS) is the main bioactive component of the cell wall of Gram-negative (G-) bacterium and is critical for the inflammatory response [7]. Therefore, it is imminent to clarify the potential mechanism of inflammatory response and develop new effective strategies for the clinical treatment of pneumonia.

Long noncoding RNAs (lncRNAs) have a molecular size of longer than 200 nucleotides (nt) and have no protein-coding potential but exhibit transcription and processing capacity [8,9]. Currently, increasing evidences have demonstrated that numerous lncRNAs are critical transcriptional or post-transcriptional modulators to regulate gene expression, thus participating in physiological and pathological processes [10]. Fast-growing number of studies have disclosed that abnormal regulation of lncRNAs is associated with various human diseases, for instance, tumors [11], cardiovascular diseases [12], neurodegeneration diseases [13], inflammatory diseases, pulmonary fibrosis [14] and lung carcinoma [15]. Small nucleolar RNA host gene 16 (SNHG16,NR_038111.1) has been found as an oncogenic lncRNA in

* Corresponding author at: Department of Pulmonary Medicine, HwaMei Hospital, University of Chinese Academy of Sciences, No.41 Northwest Street, Haishu District, 315010, Ningbo, Zhejiang Province, China.
E-mail address: YenaZhang@163.com (Y. Zhang).

https://doi.org/10.1016/j.lfs.2019.05.008
Received 17 February 2019; Received in revised form 29 April 2019; Accepted 5 May 2019
Available online 07 May 2019
0024-3205/

multiple types of human cancer such as colorectal carcinoma [16], breast carcinoma [17], bladder carcinoma [18], lung carcinoma, and so on. Of note, previous findings of Mishra et al. [19] firstly reported that SNHG16 was abnormally expressed in interstitial lung disease, involved in inflammation and host defense, and may be a therapeutic and diagnostic target for interstitial lung disease. However, the role of SNHG16 in pneumonia has not yet been elucidated.

Various evidences reveal that lncRNAs function as competitive endogenous RNAs (ceRNAs) to sponge target miRNAs via binding to miRNA response elements (MREs), and ultimately regulate cancer-related gene expression [20]. MicroRNAs (miRNAs) are short non-coding RNAs consists of 20–22 nucleotides, and negatively lead to mRNA degradation or translation repression by binding to 3′-untranslated region (3′-UTR) of mRNAs [21]. Among them, miR-146a-5p shows an inflammatory inhibition effect in a variety of cells and tissues, including human airway smooth muscle [22–25]. In particular, miR-146a has a regulatory effect on repolarization of human alveolar macrophages and secretion of inflammatory cytokines in vitro [26]. Additionally, there are predicted binding sites for miR-146a and SNHG16. Above all, it is worth exploring whether SNHG16 is able to interact with miR-146a thus playing a regulatory role in pneumonia.

In this study, we found that the serum level of SNHG16 was highly increased in patients with acute stage pneumonia. Thereby, we hypothesized that SNHG16 might regulate the advancement of pneumonia. To verify our hypothesis, pneumonia and lung injury cell model were constructed with lipopolysaccharides (LPS) treatment in human lung fibroblast cell line WI-38 as described in previous studies [27–29]. Besides, expression level change of SNHG16 in pneumonia WI-38 cell model was investigated. Furthermore, the function of SNHG16 in LPS-induced cell viability, apoptosis and inflammatory damage were analyzed. Interestingly, mechanism studies declare that SNHG16 might serve as ceRNA to increase the expression of C—C motif chemokine ligand 5 (CCL5) via directly targeting and suppressing miR-146a-5p expression, which serves as a contributor to the activation of JNK and NF-κB signaling. Our data supply the evidence for the role of SNHG16 in pneumonia by modulating miR-146a-5b/CCL5 axis, and provide new target for diagnosis and treatment of pneumonia.

2. Materials and methods

2.1. Patients and sample collection

30 patients with acute stage pneumonia (mean age, 25.5 ± 3.2 years; 24 males and 6 females) and 30 healthy individuals (mean age, 26.0 ± 2.4 years; 23 males and 7 females) from the HwaMei Hospital between June 2016 and July 2017 were recruited in this study. Exclude patients with other complications or patients who had received anti-inflammatory treatment. 3 mL of fasted peripheral venous blood was collected, centrifuged and stored at −80 °C. Each patient signed a written informed consent in advance, and the study was approved by the ethics committee of HwaMei Hospital.

2.2. Cell culture and LPS treatment

Normal human fibroblast WI-38 cell line (ATCC; Manassas, VA, USA) were incubated in Dulbecco's modified Eagle's medium (DMEM; Invitrogen, Carlsbad, CA) supplemented with 10% (v/v) fetal bovine serum, and 1% penicillin/streptomycin (Invitrogen) in a humidified atmosphere 5% CO_2 at 37 °C. 2×10^5 cells were plated in 6-well plates and incubated 24 h. LPS in different concentrations (5, 10 and 20 μg/ml) was added in medium for 12 h to establish injury model [28–30].

2.3. Cell transfections

The full length of SNHG16 was amplified from human cDNA by PCR (forward primer 5′- CCAAGCTTATGCCAGATGGGATCAGCAC -3′ and reverse primer 5’-CCGCTCGAGCTTGGTGAGTCAACACTGGGT -3′) and inserted into pcDNA3.1 vector (Invitrogen, USA). The siRNA of SSNHG16, miR-146a-5p mimic, inhibitor, and the respective negative controls were synthesized and purchased from Applied Biological Materials (GenePharma, Shanghai, China). Cell transfections were executed using Lipofectamine 2000 reagent (Invitrogen), according to the manufacturer's instructions.

2.4. RNA extraction and qRT-PCR

Total RNA was isolated using Trizol reagent (Invitrogen) and next synthesized to first-strand cDNA using PrimeScript™ RT reagent Kit (Takara, Japan). qRT-PCR was performed using SYBR Prime Script RT-PCR Kits (Takara, Japan). miR-146a-5p and other mRNA expression levels were normalized to U6 and β-actin by $2^{-\Delta\Delta Ct}$ method, respectively. Primers are detailed as follows: SNHG16, forward (F) 5′-CCCA AGCTTGCGTTCTTTTCGAGGTCGGC-3′; reverse (R) 5′-CCGGAATT CTGACGGTAGTTTCCCAAGTT-3′; miR-146a-5p, stem loop: 5’-GTCGT ATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACTTCTCC; F 5′-CCGATGTGTATCC TCAGCTTTG-3′; R 5′-GCTGAAGAACTGAATTTC AGAGGTC-3′; CCL5, F 5′-AATCTTGCAGTCGTGTTTGTCA-3′; R 5′-AGC TCATCTCCAAATAGTTG ATGT-3′; IL-6, F 5′-GAAATCGTGGAAAT GAG-3′;R 5′-TAGGTTTGCCGAGTA GA-3′; IL-1β, F 5′-GCCCTAAACAG ATGAAGTGCTC-3′; R 5′-GAACCAGC ATCTTCCTCAG-3′; TNF-α, F 5′-GCCAATGGCATGGATCTCAAAG-3′; R 5′-CA GAGCAATGACTCCAA AGT-3′; U6, F 5′-TGCGGGTGCTCGCTTCGGCAGC -3′; R 5′-CCAGTGCA GGGTCCGAGGT-3′; β-actin, F 5′-TGGAATCCTG TGGCATCCATGA AAC-3′;R 5′-ACGCAGCTCAGTAACAGTCCG-3′.

2.5. Cell viability assay

The cell viability was assessed using the CCK8 proliferation detection kit (Dojindo, Tokyo, Japan). Briefly, a total of approximately 5×10^3 cells were seeded in 96-well plates, after LPS stimulation, 20 μl CCK-8 solution was added to each well and cultivated for 1 h. The absorbance of the reaction system was measured spectrophotometrically at 450 nm.

2.6. Detection of cell apoptosis

Flow cytometry analysis for apoptosis was performed with Annexin V FITC- propidium iodide (PI) staining assay (Invitrogen, California, USA) based on the manufacturer's instruction.

2.7. Enzyme-linked immunosorbent assay (ELISA)

WI-38 cell culture supernatant in 24-well plates was collected, and the productions of inflammatory cytokines were measured by ELISA kits (R&D Systems, Minneapolis, MN, USA) as instructed and quantified by normalization to protein concentrations.

2.8. Western blotting

Cells were lysed with lysis buffer containing protease inhibitors (50 mM Tris-HCl pH 8, 50 mM NaCl, 0.5% NP-40). Equal amounts of protein were resolved by Sodium Dodecyl Sulfate-PolyAcrylamide Gel Electrophoresis (SDS-PAGE) gels, and transferred to PVDF membranes. After blocking with 5% nonfat milk, the membranes were then immunoblotted with primary antibodies against CCL5, IL-6, IL-1β, TNF-α, Bcl-2, Bax, cleaved-caspase-3, cleaved-caspase-9, JNK,p-JNK , c-Jun, p-c-Jun , p65 , p-p65 , IκBα , p-IκBα and β-actin (Santa Cruz, USA) at a dilution of 1:1000 overnight at 4 °C. Following washing with TBST, the membranes were exposed to HRP-conjugated goat anti-rabbit (1:5000, Santa Cruz), secondary antibodies and signals were detected with ECL detection system.

2.9. Luciferase reporter assay

WI-38 cells were cultured into 24-well plates and transfected with pmirGLO Dual-luciferase vector (SNHG16 WT, SNHG16 MUT, CCL5 WT and CCL5 MUT) together with miR-146a-5p mimics or negative control (NC mimics). After 24 h, luciferase activity was measured by the dual-luciferase reporter assay system (Promega, Madison, WI, USA).

2.10. RNA immunoprecipitation (RIP) assay

RIP experiment was conducted using Magna RIP RNA-binding protein immunoprecipitation kit (Millipore, Billerica, MA, USA) and the Ago2 antibody (Abcam, Cambridge, MA, USA) according to manufacturer's instruction. The expression of SNHG16 was analyzed by qRT-PCR. Normal mouse IgG (Abcam, USA) anti-SNRNP70 (Abcam, USA) were respectively used as negative control and positive control.

2.11. RNA-pull down assay

Purified RNAs were respectively labeled and transcribed with Biotin RNA Labeling Mix (Roche, Switzerland) and T7 RNA polymerase (Ambion Life) in vitro, and purified withan RNeasy Plus Mini Kit (Qiagen, Valencia, CA,USA) and DNase I (Qiagen). Purified RNAs The positive control, negative control, and biotinylated RNAs were mixed and incubated with AGS cell lysates. Then, magnetic beads were added to each binding reaction, and incubated at room temperature. RNA complexes bound to these beads were eluted and extracted for qRT-PCR analysis.

2.12. RNA fluorescent in situ hybridization(RNA-FISH)

Cy3-labeled SNHG16 and DAPI-labeled U6 probes were designed and synthesized by RiboBio (Guangzhou, China). A fluorescent in situ hybridization kit was used to perform RNA-FISH assay according to the manufacturer's protocol (Thermo Fisher).

2.13. Statistical analysis

All quantitative data were presented as mean ± SD and analyzed using SPSS 19.0 software (SPSS Inc., Chicago, IL, USA). The comparison significances were respectively evaluated by a two-sided Student's *t*-test for two groups and one-way ANOVA test for three or more groups. All experiments were repeated at least three times. $P < 0.05$ was considered as statistically significant.

3. Results

3.1. SNHG16 expression was up-regulated in serum of pneumonia patients

To investigate the expression of SNHG16 in pneumonia, qRT-PCR analysis was conducted. The results showed that SNHG16 was significantly increased in acute stage pneumonia than that healthy control (Fig. 1A). Hence, we speculated that SNHG16 might play a potential role in regulating acute pneumonia development.

3.2. LPS stimulation induced cell apoptosis and inflammation injuries in WI-38 cells

To examine the functions of LPS on WI-38 cells, different concentrations of LPS (5, 10, and 20 μg/ml) were used to induce cell injury, and results of CCK-8 assay demonstrated that cell viability was obviously reduced with increasing LPS concentration (Fig. 1B). Furthermore, the cell apoptosis was significantly increased with increasing concentration of LPS (Fig. 1C). Meanwhile, LPS induced the decreased protein levels of anti-apoptotic marker Bcl-2, while increased the protein levels of pro-apoptotic markers including Bax, cleaved caspase-3, and cleaved caspase-9 (Fig. 1D). By performing ELISA assay, LPS evidently increased the secretion of pro-inflammatory cytokines (IL-6, IL-1β and TNF-α) (Fig. 1E). In agreement, qRT-PCR and Western Blotting assays indicated that the mRNA and protein levels of the above-mentioned pro-inflammatory factors were also significantly decreased (Fig. 1F–G). Since 10 μg/ml LPS contributed to an obvious reduction in cell viability while significant increase in cellular inflammatory damage, 10 μg/ml was selected for the following experimental conditions. These data suggest that LPS induced acute pneumonia WI-38 cell model is successfully constructed.

3.3. SNHG16 was higher expressed in LPS-induced WI-38 cells

SNHG16 was reported to serve as an oncogenic lncRNA in cancers, and also could cause inflammatory response in interstitial lung disease and neonatal sepsis [19,31]. To profile the expression of SNHG16 in LPS-injured WI-38 cells, qRT-RCR analysis was evaluated and the results demonstrated that SNHG16 expression was significantly elevated at different degrees in WI-38 cells with LPS treatments at various levels.

3.4. Inhibition of SNHG16 alleviated cell apoptosis and inflammation injuries in LPS-induced WI-38 cells

To examine the possible functions of SNHG16 in LPS-induced inflammatory injuries, WI-38 cells were transfected with siRNA targeting SNHG16. qRT-PCR assay was applied to verify the transfection efficiency. More importantly, the data demonstrated that SNHG16 expression was dramatically inhibited by si-SNHG16#1 and si-SNHG16#2 transfection compared with si-NC group, and si-SNHG16#2 was selected for subsequent experiments because of better knock-down effect (Fig. 2B). As a result, knockdown of SNHG16 significantly enhanced cell viability and the protein level of Bcl-2, whereas decreased apoptotic cell rates, and the protein of Bax, cleaved Caspase-3 and cleaved Caspase-9 (Fig. 2C–E) in response to LPS treatment. Meanwhile, knockdown of SNHG16 also reduced the production of IL-6 IL-1β and TNF-α in LPS-induced WI-38 cells (Fig. 2F–H). Collectively, above findings indicate that SNHG16 can increase inflammatory injuries of LPS-induced WI-38 cells.

3.5. SNHG16 suppressed miR-146a-5p expression by functioning as a sponge in LPS-induced WI-38 cells

There have been studies showing that lncRNA may be a kind of ceRNA of miRNA [32]. The subcellular localization of lncRNA is closely associated with its biological effects and potential molecular roles. Thus, RNA-FISH assay was performed to detect the subcellular distribution of SNHG16. The results showed that most of punctate patterns were abundant in the cytoplasm, while minority in the nucleus (Fig. 3A), which was also verified by nucleo-cytoplasmic separation experiment (Fig. 3B). Aiming to elucidate the exact mechanisms underlying the function of SNHG16, we searched its potential targets using Starbase database (http://starbase.sysu.edu.cn/starbase2/index.php), a putative interaction between SNHG16 and miR-146a-5p was found and binding sites of wild-type (SNHG16-WT) and mutant-type (SNHG16-MUT) was shown (Fig. 3C). Luciferase reporter assay demonstrated that SNHG16-WT and miR-146a-5p mimics co-transfection significantly reduced luciferase activity, while SNHG16-MUT and miR-146a-5p mimics co-transfection failed to affect luciferase activity in WI-38 cells (Fig. 3D). The interaction between miR-146a-5p and SNHG16 was further validated in RIP assay and the results indicated that SNHG16 exerted more significant enrichment in the Ago2-containing miRNA ribonucleoprotein complexes (miRNPs) than the control IgG immunoprecipitates (Fig. 3E). Meantime, biotin-labeled pull-down assay demonstrated that SNHG16 was efficiently pulled down by bio-miR-146a-5p (Fig. 3F), but not bio-miR-146a-5p MUT. Consistently, qRT-PCR analysis indicated that knockdown of SNHG16 upregulated

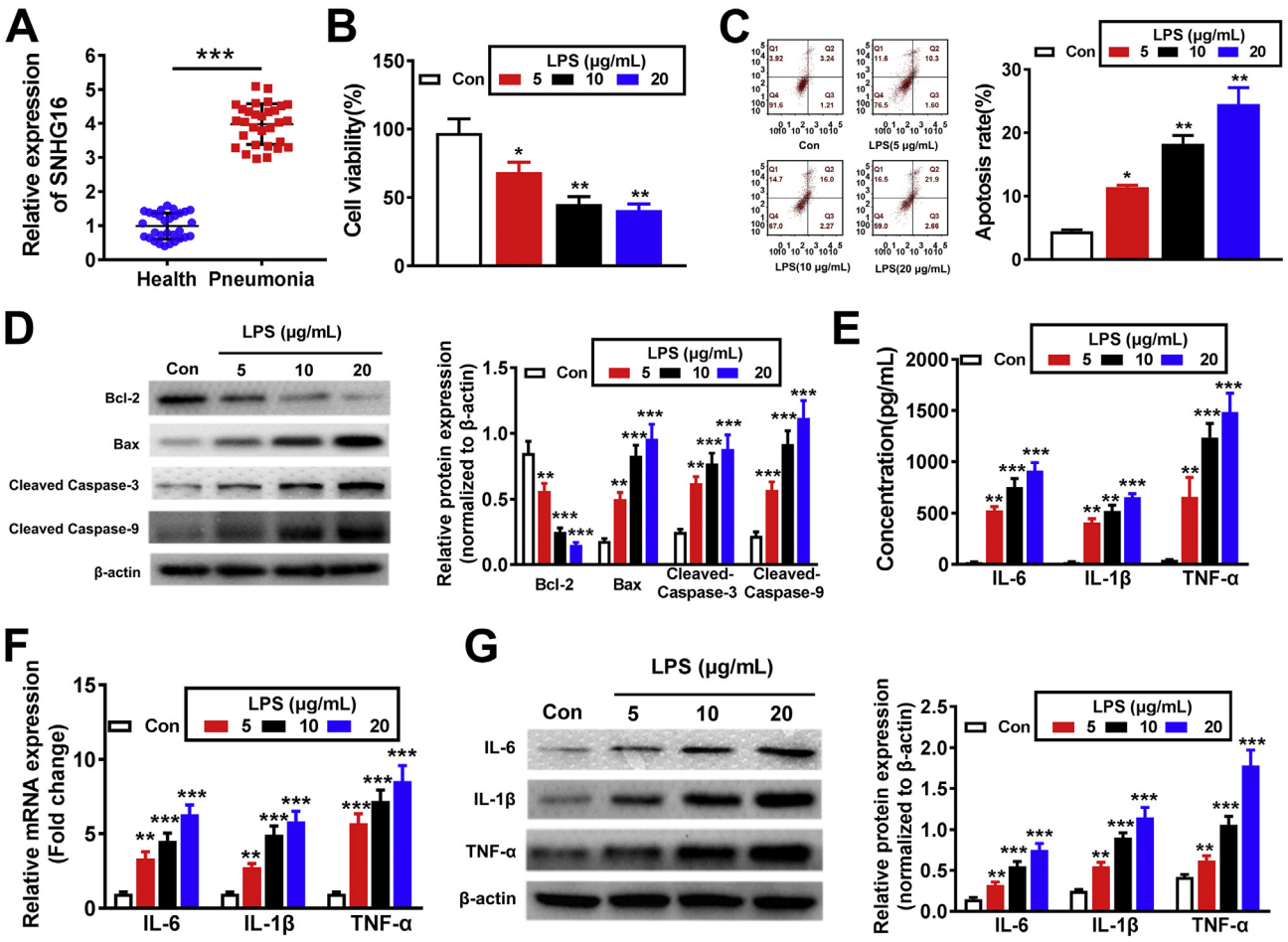

Fig. 1. Expression of SNHG16 in the clinical cases and LPS induces inflammatory injury in WI-38 cells. (A) SNHG16 expression in serum of acute stage pneumonia patients and healthy controls was detected by qRT-PCR. (B) The effect of different concentrations of LPS (5, 10, and 20 μg/ml) on cell viability was estimated by CCK-8 assay. (C-D) The effect of LPS on cell apoptosis was evaluated by (C) flow cytometry and (D) Western blotting. (E–G) The expression levels of IL-6, IL-1β, and TNF-α were evaluated by (E) ELISA, (F) qRT-PCR, and (G) Western blotting after treatment with LPS. $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$.

miR-146a-5p expression, while miR-146a-5p inhibitor considerably enhanced SNHG16 expression in WI-38 cells (Fig. 3G). Moreover, the expression level of miR-146a-5p in serum of acute stage pneumonia patients was significantly decreased compared with healthy control (Fig. 3H), and the level of SNHG16 and miR-146a-5p exhibited a dramatically negative correlation confirmed by Spearman's correlation analysis (Fig. 3I). Furthermore, expression level of miR-146a-5p was signally reduced with LPS induction in WI-38 cells in a concentration-dependent manner (Fig. 3J). On the basis of above findings, SNHG16 might exert an effect on miR-146a-5p deregulation through functioning as a sponge.

3.6. CCL5 was a target gene of miR-146a-5p

To ascertain the target gene through which miR-146a-5p exerts its function , Targetscan database (http://www.targetscan.org/) predicted that CCL5 was a putative target gene of miR-146a-5p. A putative interaction between miR-146a-5p and CCL5-WT and the modified binding sequence are detailed in Fig. 4A. Results of dual luciferase reporter assay suggested that the luciferase activity of cells with CCL5-WT transfection was significantly decreased by miR-146a-5p mimics, while no alter in CCL5-MUT transfected group (Fig. 4B). To further confirm this result, miR-146a-5p was overexpressed or inhibited in WI-38 cells and the transfection efficiency was evaluated by qRT-PCR (Fig. 4C). Results of qRT-PCR and western blotting analysis proved that miR-146a-5p mimics significantly enhanced, while miR-146a-5p inhibitor significantly reduced the expression of CCL5 at mRNA and protein level (Fig. 4D–E). The above data suggested that miR-146a-5p was directly binding to CCL5. Moreover, the serum CCL5 expression in acute stage pneumonia patients was obviously higher than healthy control (Fig. 4F). Next, Spearman's correlation analysis proved that the expressions of miR-146a-5p and CCL5 were inversely correlated, while SNHG16 and CCL5 were positively correlated in acute stage pneumonia patients (Fig. 4G and H). In addition, CCL5 mRNA was distinctly increased with LPS induction in WI-38 cells in a concentration-dependent manner (Fig. 4I). Altogether, the above results proved that CCL5 is a target gene of miR-146a-5p and positively regulated by SNHG16.

3.7. Inhibition of SNHG16 inhibits LPS-induced apoptosis and inflammatory damage via miR-146a-5p in LPS-injured WI-38 cells

To explore the regulatory relationship between SNHG16 and miR-146a-5p, SNHG16 was down-regulated by its siRNA, and miR-146a-5p expression was further knockdown by its inhibitor in WI-38 cells. As expected, si-SNHG16 improved cell viability and reduced apoptosis, whereas the effect of SNHG16 silencing was largely abrogated by the concomitant inhibition of miR-146a-5p (Fig. 5A–B). Congruously, the function of si-SNHG16 on increasing Bcl-2 and decreasing the Bax, cleaved-caspase-3 and cleaved-caspase-9 protein levels was abolished by co-transfection with miR-146a-5p inhibitor (Fig. 5C). Furthermore, decreased inflammatory factors (IL-6, IL-1β and TNF-α) by SNHG16 inhibition was also reversed by miR-146a-5p inhibition (Fig. 5D–F).

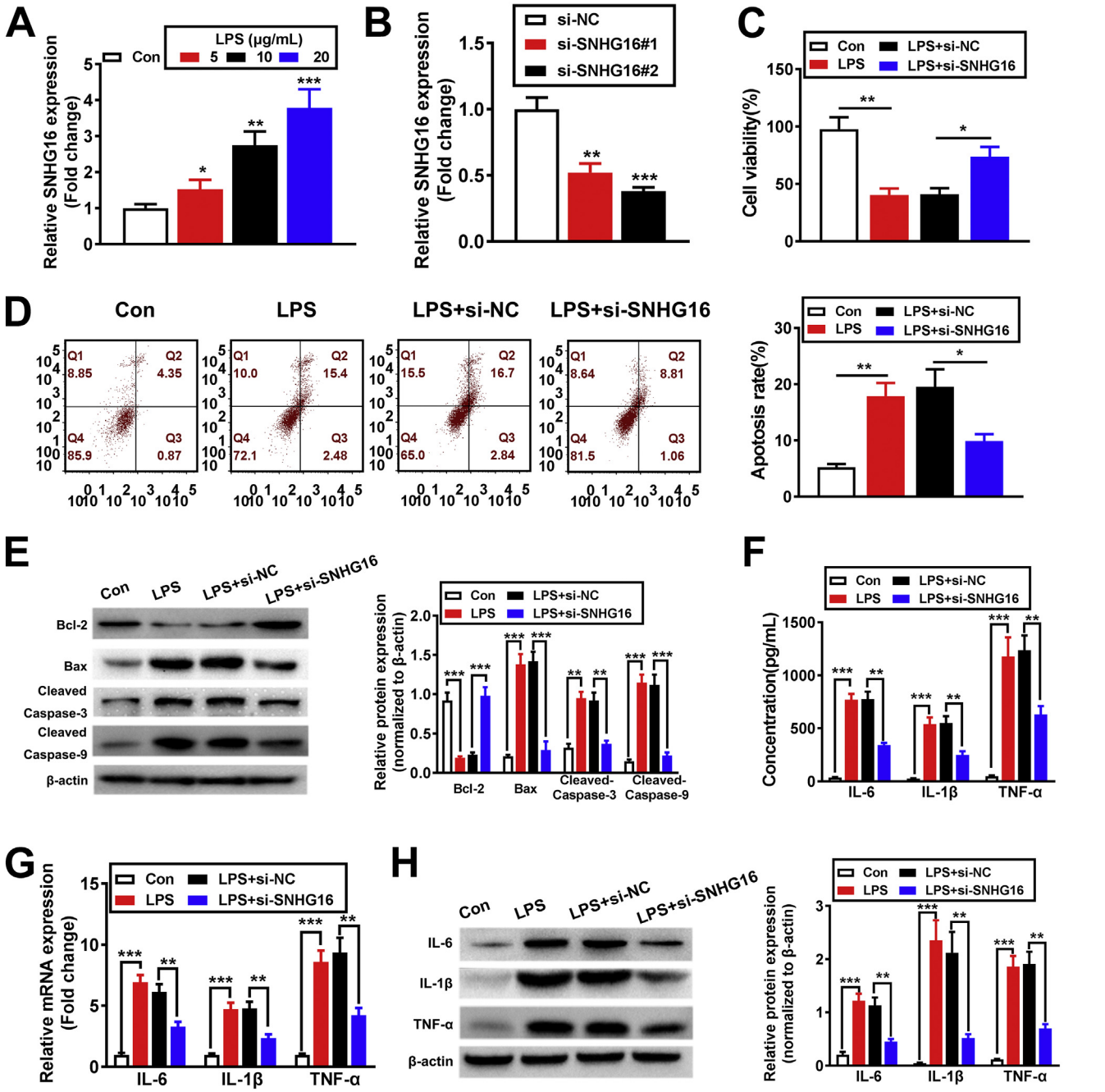

Fig. 2. Inhibition of SNHG16 alleviated cell apoptosis and inflammation injuries in LPS-induced WI-38 cells. (A) SNHG16 expression was detected by qRT-PCR. (B) The transfected efficiency by si-SNHG16 was analyzed by qRT-PCR. (C)The effect of SNHG16 knockdown on cell viability was analyzed by CCK-8 assay. (D–E) The effect of SNHG16 knockdown on cell apoptosis was evaluated by (D) flow cytometry and (E) Western blotting. (F–H) The effect of SNHG16 knockdown on the expression levels of IL-6, IL-1β, and TNF-α were analyzed by (F) ELISA, (G) qRT-PCR, and (H) Western blotting after treatment with LPS. $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$.

Above results demonstrated that SNHG16 aggravate LPS-induced WI-38 cell injuries through targeting miR-146a-5p.

3.8. SNHG16 regulated JNK and NF-κB pathways in LPS-induced WI-38 cells

For the purpose of further validating the regulatory function of SNHG16, we silenced SNHG16 in WI-38 cells, and a significantly reduced CCL5 expression was observed, whereas the concomitant inhibition of miR-146a-5p prevented this reduction. JNK and NF-κB pathways are well-accepted to participate in the regulation of inflammatory response, we found that the activation of JNK and NF-κB signaling pathways were positively correlated with the expression level of CCL5, both of which were negatively regulated by miR-146a-5p whose function was antagonized by SNHG16(Fig. 6A–B). More narrowly, LPS treatment markedly led to the increased expression levels of p-JNK, p-c-Jun, p-p65, and p-IκBα in WI-38 cells while this increase effect was obviously inhibited by si-SNHG16. Moreover, the inhibited effect of SNHG16 knockdown on decreased proteins mentioned above was significantly rescued by miR-146a-5p inhibitor in LPS-treated WI-38 cells. These data indicate that SNHG16 support LPS-induced activation of JNK and NF-κB pathways through regulating miR-146a-5p/

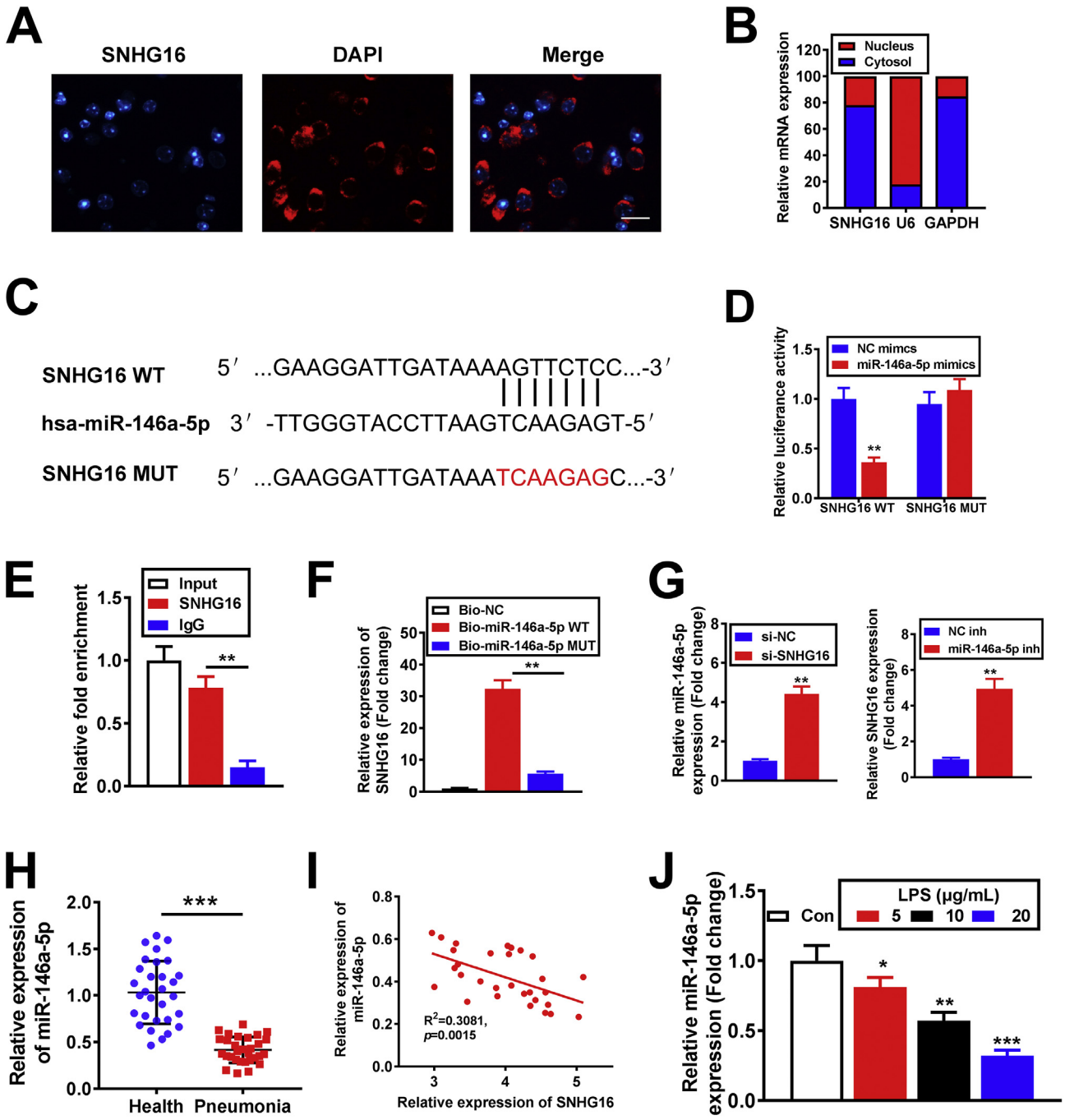

Fig. 3. SNHG16 suppressed miR-146a-5p expression by functioning as a sponge in LPS-induced WI-38 cells. (A) Localization of SNHG16 by RNA-FISH in WI-38 cells. Nuclei are stained red (DAPI) and SNHG16 is stained blue (Scale bar = 50 μM). (B) SNHG16 expression in Nucleus and Cytoplasm was detected by qRT-PCR. (C) Putative miR-146a-5p binding sequence and mutation sequence of SNHG16 mRNA were as shown. (D) Luciferase reporter assays were used to confirm the direct target between SNHG16 and miR-146a-5p. (E) Association of miR-146a-5p and SNHG16 with AGO2 was performed by Immunoblotting assays. RNA levels were presented as fold enrichment in Ago2 relative to IgG immunoprecipitates (lower panel). (F) qRT-PCR was used to detect SNHG16 expression in the sample pulled down by biotinylated miR-146a-5p WT and miR-146a-5p MUT probe. (G) miR-146a-5p and SNHG16 expression levels were detected by qRT-PCR. (H) miR-146a-5p expression in serum of acute stage pneumonia patients and healthy controls was analyzed by qRT-PCR. (I) The correlation analysis between SNHG16 expression and miR-146a-5p expression in acute stage pneumonia patients (n = 30) was performed by Spearman's rank correlation analysis. (J) miR-146a-5p expression was detected by qRT-PCR. $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

CCL5 axis in WI-38 cells.

4. Discussion

Pneumonia is a main infectious disease with the world's leading mortality and morbidity rates, particularly in children and seniors. It is well known that pneumonia is associated with inflammatory stimulation from microorganisms. Identification of key lncRNAs facilitates to the development of effective therapeutic targets for pneumonia. Recently, there is abundant evidence to suggest that lncRNAs are disordered and participate in regulating pneumonia [28,30]. Therefore, exploring lncRNA signature might be valuable for diagnosis and

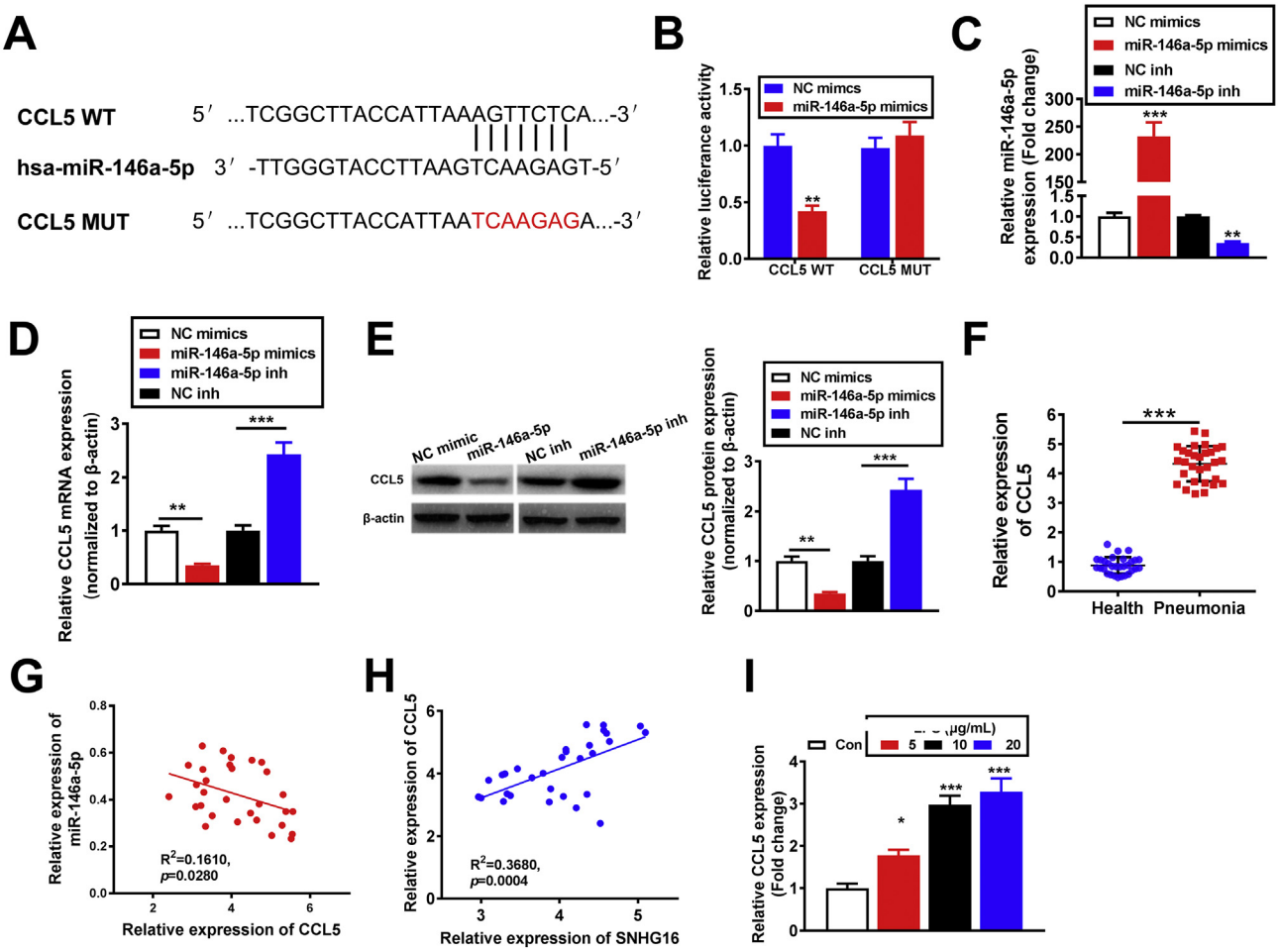

Fig. 4. CCL5 was a target gene of miR-146a-5p. (A) Putative miR-146a-5p binding sequence and mutation sequence of CCL5 mRNA were as shown. (B) Luciferase reporter assays were used to confirm the direct target between CCL5 and miR-146a-5p. (C) The transfection efficiency by miR-146a-5p mimics and inhibitor was analyzed by qRT-PCR. (D–E) The expression levels of CCL5 was analyzed by (D) qRT-PCR and (E) Western blotting. (F) CCL5 expression in serum of acute stage pneumonia patients and healthy controls was analyzed by qRT-PCR. (G–H) The correlation analysis (G) between CCL5 and miR-146a-5p expression, the correlation analysis (H) between CCL5 and SNHG16 expression in acute stage pneumonia patients (n = 30) were performed by Spearman's rank correlation analysis. (I) CCL5 expression was analyzed by qRT-PCR. $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$.

treatment of pneumonia. SNHG16 was originally reported to involve in the development of various cancers as an oncogene. A following study indicated that SNHG16 served as an independent prognostic marker which can promote proliferation and suppress apoptosis by recruiting EZH2 further epigenetically silencing p21 in bladder cancer [18]. Meanwhile, SNHG16 contributes to the tumorigenesis by serving as an endogenous 'sponge' to regulate miR-216A-5p/ZEB1 in cervical cancer [33]. Not only in cancer, current evidence suggests that SNHG16 also has a significant impact on regulating inflammation response. SNHG16 can regulate miR-15a/16 cluster to upregulate TLR4 and promote LPS-induced inflammation in RAW264.7 cells [31]. In the present study, SNHG16 was upregulated in acute stage pneumonia patients and LPS-induced WI-38 cells. SNHG16 inhibition decreased cell injury through increasing cell viability, and alleviating apoptosis and inflammation in LPS-induced WI-38 cells. Further mechanism investigations revealed that SNHG16 could negatively regulated miR-146a-5p expression but positively modulated CCL5 expression via acting as a sponge of miR-146a-5p. Besides, the effect of SNHG16 silencing on cell apoptosis and inflammation in LPS-induced WI-38 cells was abolished by co-transfection with miR-146a-5p inhibitor. Additionally, SNHG16 blocked LPS-activated of JNK and NF-κB pathways which were reversed by miR-146a-5p.

It has been demonstrated that lncRNAs affect disease progression by competitively binding miRNAs to regulate target genes expression via the ceRNA mechanism [34]. Our data confirmed that SNHG16 was negatively correlated with miR-146a-5p, and SNHG16 affected apoptosis and inflammation of LPS-induced WI-38 cells by regulating miR-146a-5p. Previous studies have shown that miR-146a negatively regulates inflammatory factors (IL-6 and IL-8) associated with senescence and decreases lipid accumulation and inflammation induced by oxidized low-density lipoprotein through targeting TLR4 [35,36]. Moreover, Ye et al. revealed that miR-146a inhibited TNFα production and the activation of TLR4/NF-κB in high glucose-induced primary human retinal microvascular endothelial cells [19]. In our study, miR-146a-5p was firstly found to be abnormally dysregulated in pneumonia patients and could alleviate inflammation injury in LPS-injured pneumonia cell model.

What is more, CCL5 (regulated upon activation normal T cell expressed and secreted, RANTES) which was initially known as a product of activated T cells, was confirmed as a target gene of miR-146a-5p by Targetscan prediction and functional experiments in our data. Now CCL5 is diffusely considered to be an inflammatory chemokine which involved in various inflammatory diseases, e.g., inflammatory bowel disease, rheumatoid arthritis, cardiac dysfunction with progressive inflammation, etc. [37–40]. Notably, a recent study illustrated that CCL5 silencing could inhibit inflammatory injuries in LPS-induced WI-38 cells from through mediating JNK and NF-κB signaling. Normally, JNK and NF-κB pathways have been deemed to regulate pneumonia. For

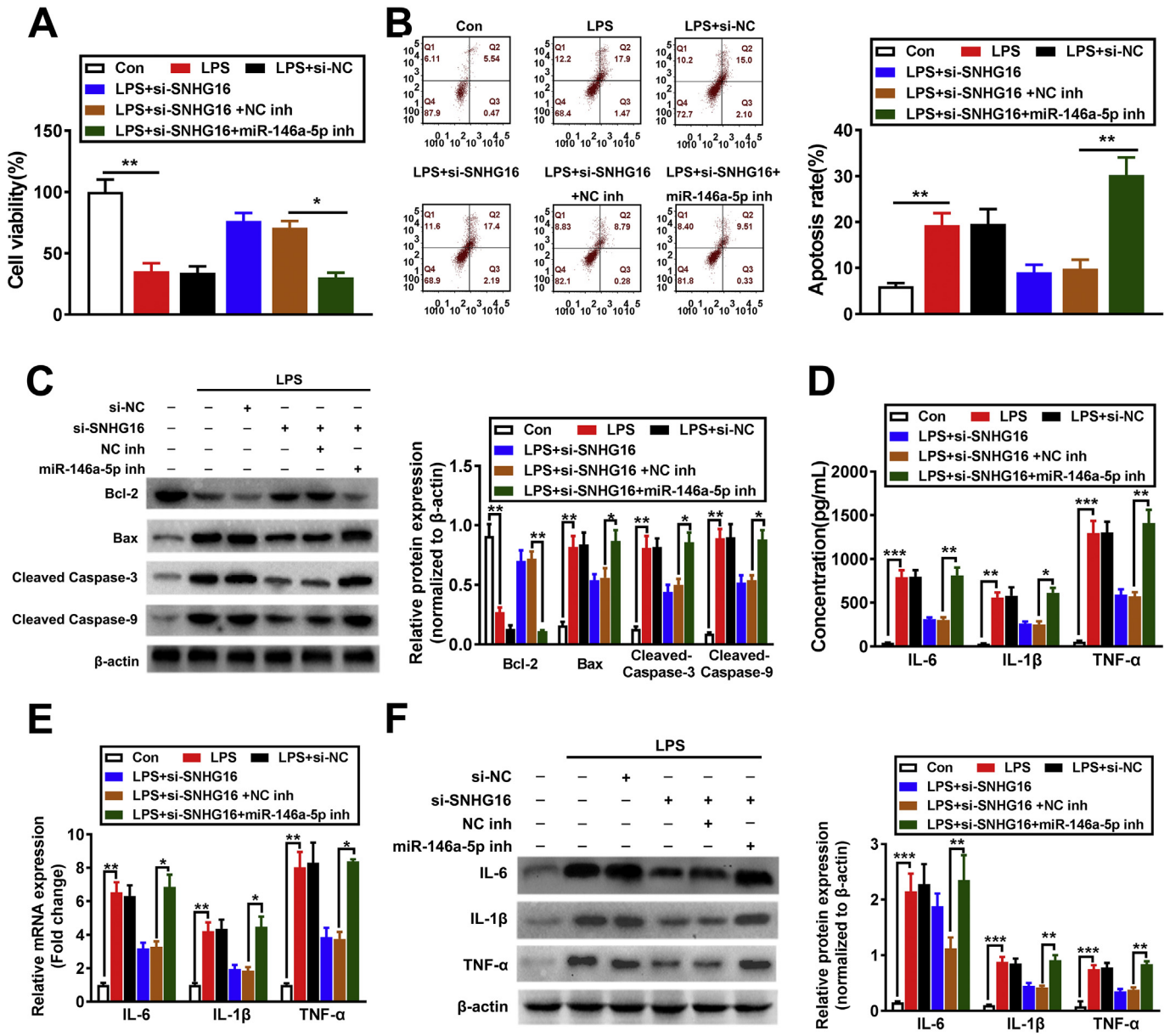

Fig. 5. Downregulation of SNHG16 inhibits LPS-induced apoptosis and inflammatory damage via miR-146a-5p in LPS-induced WI-38 cells. (A) Cell viability was analyzed in si-NC, si-SNHG16 transfected or si-SNHG16 + NC inhibitor or si-SNHG16 + miR-146a-5p inhibitor co-transfected WI-38 cells after LPS treatment by CCK-8 assay. (B—C) Cell apoptosis in si-NC, si-SNHG16 transfected or si-SNHG16 + NC inhibitor or si-SNHG16 + miR-146a-5p inhibitor co-transfected WI-38 cells after LPS treatment was evaluated by (B) flow cytometry and (C) Western blotting. (D-F) The expression levels of IL-6, IL-1β, and TNF-α were analyzed by (D) ELISA, (E) qRT-PCR, and (F) Western blotting in si-NC, si-SNHG16 transfected or si-SNHG16 + NC inhibitor or si-SNHG16 + miR-146a-5p inhibitor co-transfected WI-38 cells after treatment with LPS. $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$.

instance, Guo et al. suggested that miR-1247 might regulate acute pneumonia progression through activating JNK and NF-κB signaling [41]. Considering the important role of JNK and NF-κB in pneumonia, SNHG16 was knockdown for function and mechanism investigation. SNHG16 inhibition reduced LPS-induced activation of JNK and NF-κB pathways, this result explained the underlying mechanism by which SNHG16 worked.

Taken together, our study for the first time uncovered that SNHG16 inhibition attenuated LPS-induced WI-38 cell injury via modulating miR-146a/CCL5 axis, which may thrown new perspectives for diagnostic and therapeutic approach to pneumonia.

Acknowledgements

Not applicable.

Funding

No funding was received.

Availability of data and materials

The datasets analyzed in the present study are available from the corresponding author on reasonable request.

Ethics approval and consent to participate

The present study was approved by the Ethical Committee of HwaMei Hospital (Ningbo, China).

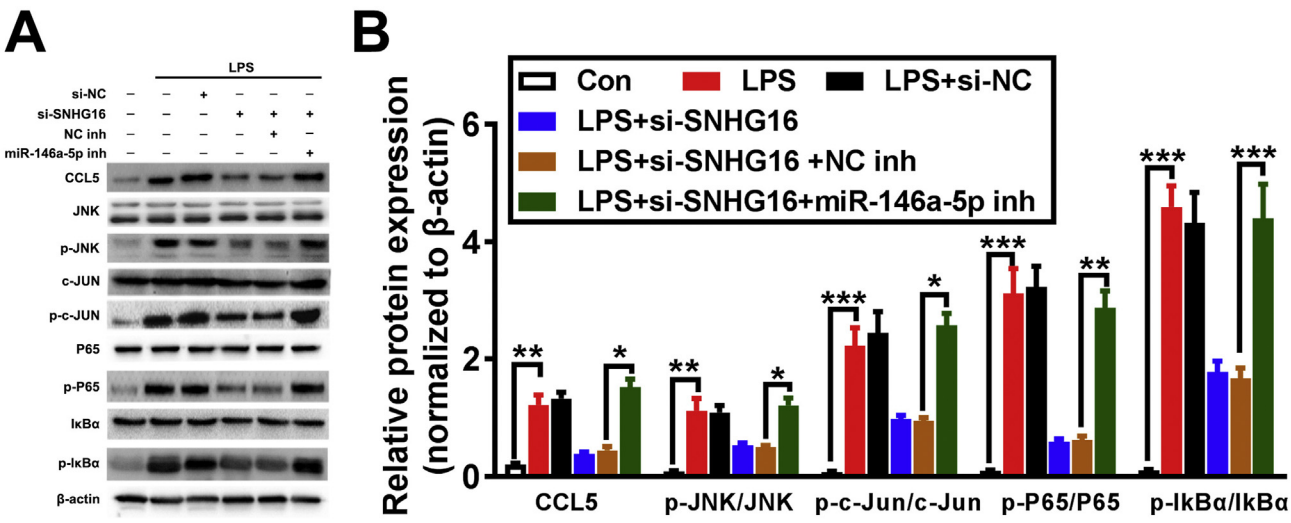

Fig. 6. SNHG16 regulated JNK and NF-κB signaling pathways in LPS-induced WI-38 cells. (A) Representative western blotting results for CCL5, JNK, p-JNK, c-JUN, p-c-JUN, p65, p-p65, IκBα and p-IκBα protein expression from si-NC, si-SNHG16 transfected or si-SNHG16 + NC inhibitor or si-SNHG16 + miR-146a-5p inhibitor co-transfected WI-38 cells after LPS treatment. (B) Quantitative analysis of proteins was obtained by using β-actin as a control. $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$.

Competing interests

None.

References

[1] C. Cillóniz, et al., Community-acquired pneumonia related to intracellular pathogens, 42 (9) (2016) 1374–1386.
[2] M.N. Lutfiyya, et al., Diagnosis and treatment of community-acquired pneumonia, 73 (3) (2006) 442–450.
[3] M.J.A.P. Korppi, Diagnosis and treatment of community-acquired pneumonia in children, 101 (7) (2012) 702–704.
[4] A.F. Simonetti, et al., Management of community-acquired pneumonia in older adults, 2 (1) (2014) 3–16.
[5] T. Wardlaw, et al., Pneumonia: the leading killer of children, 368 (9541) (2006) 1048–1050.
[6] M. Rojas, et al., Endotoxin-induced lung injury in mice: structural, functional, and biochemical responses, Am. J. Physiol. Lung Cell. Mol. Physiol. 288 (2) (2005) L333.
[7] D. Didier, R. Jean-Damien, Acute lung injury and bacterial infection, Clin. Chest Med. 26 (1) (2005) 105.
[8] J. Ponjavic, et al., Functionality or transcriptional noise? Evidence for selection within long noncoding RNAs, Genome Res. 17 (5) (2007) 556.
[9] C.P. Ponting, et al., Evolution and functions of long noncoding RNAs, Cell 136 (4) (2009) 629–641.
[10] K.C. Wang, H.Y. Chang, Molecular mechanisms of long noncoding RNAs, Mol. Cell 43 (6) (2011) 904.
[11] R. Spizzo, et al., Long non-coding RNAs and cancer: a new frontier of translational research? Oncogene 31 (43) (2012) 4577–4587.
[12] S. Uchida, S. Dimmeler, Long noncoding RNAs in cardiovascular diseases, Circ. Res. 116 (4) (2015) 737.
[13] P. Wan, et al., The role of long noncoding RNAs in neurodegenerative diseases, 54 (3) (2017) 2012–2021.
[14] X. Song, et al., Analysing the relationship between lncRNA and protein-coding gene and the role of lncRNA as ceRNA in pulmonary fibrosis, J. Cell. Mol. Med. 18 (6) (2014) 991–1003.
[15] T. Gutschner, et al., The noncoding RNA MALAT1 is a critical regulator of the metastasis phenotype of lung Cancer cells, Cancer Res. 73 (3) (2013) 1180–1189.
[16] L.L. Christensen, et al., SNHG16 is regulated by the Wnt pathway in colorectal cancer and affects genes involved in lipid metabolism, 10 (8) (2016) 1266–1282.
[17] C. Cai, et al., SNHG16 contributes to breast cancer cell migration by competitively binding miR-98 with E2F5, 485 (2) (2017) 272–278.
[18] X. Cao, et al., LncRNA-SNHG16 predicts poor prognosis and promotes tumor proliferation through epigenetically silencing p21 in bladder cancer, Cancer Gene Ther. 25 (1–2) (2017) 1.
[19] S. Mishra, et al., Integrated analysis of non-coding RNAs for the identification of promising biomarkers in interstitial lung diseases, Gene Reports 11 (2018) 87–93.
[20] B.D. Adams, et al., Targeting noncoding RNAs in disease, J. Clin. Investig. 127 (3) (2017) 761–771.
[21] M. Lagosquintana, et al., Identification of novel genes coding for small expressed RNAs, Science 294 (5543) (2001) 853–858.
[22] E.T. Osei, et al., miR-146a-5p plays an essential role in the aberrant epithelial–fibroblast cross-talk in COPD, 49 (5) (2017) 1602538.
[23] E.-A. Ye, JJJMoi Steinle, miR-146a attenuates inflammatory pathways mediated by TLR4/NF-κB and TNFα to protect primary human retinal microvascular endothelial cells grown in high glucose, 2016 (2016).
[24] Y. Chen, et al., MicroRNA-146a-5p negatively regulates pro-inflammatory cytokine secretion and cell activation in lipopolysaccharide stimulated human hepatic stellate cells through inhibition of toll-like receptor 4 signaling pathways, 17 (7) (2016) 1076.
[25] B.S. Comer, et al., MicroRNA-146a and microRNA-146b expression and anti-inflammatory function in human airway smooth muscle, 307 (9) (2014) (L727-L34).
[26] Y. Yang, et al., Regulatory effects of miR-155 and miR-146a on repolarization and inflammatory cytokine secretion in human alveolar macrophages in vitro, J. Immunopharmacol. 38 (6) (2016) 502–509.
[27] M.T. Cushion, et al., Pneumocystis carinii: growth variables and estimates in the A549 and WI-38 VA13 human cell lines, Exp. Parasitol. 60 (1) (1985) 43–54.
[28] M. Liu, et al., Long noncoding RNA HAGLROS regulates cell apoptosis and autophagy in lipopolysaccharides-induced WI-38 cells via modulating miR-100/NF-κB axis, Biochem. Biophys. Res. Commun. 3 (2018) 589–596.
[29] D. Bai, et al., The effect of down-regulation of CCL5 on lipopolysaccharide-induced WI-38 fibroblast injury: a potential role for infantile pneumonia, 21 (5) (2018) 449.
[30] D. Zhu-ge, et al., Knockdown CRNDE alleviates LPS-induced inflammation injury via FOXM1 in WI-38 cells, Biomed. Pharmacother. 103 (2018) 1678–1687.
[31] W. Wang, et al., LncRNA SNHG16 reverses the effects of miR-15a/16 on LPS-induced inflammatory pathway, Biomed. Pharmacother. 106 (2018) 1661–1667.
[32] J. Liz, M.J.B.B.A. Esteller, lncRNAs and microRNAs with a role in cancer development, 1859 (1) (2016) 169–176.
[33] H. Zhu, et al., SNHG16/miR-216-5p/ZEB1 signal pathway contributes to the tumorigenesis of cervical cancer cells, Arch. Biochem. Biophys. 637 (2018) 1–8.
[34] F.A. Karreth, PPJCd Pandolfi, ceRNA cross-talk in cancer: when ce-bling rivalries go awry, 3 (10) (2013) 1113–1121.
[35] D. Bhaumik, et al., MicroRNAs miR-146a/b negatively modulate the senescence-associated inflammatory mediators IL-6 and IL-8, 1 (4) (2009) 402.
[36] K. Yang, et al., MiR-146a inhibits oxidized low-density lipoprotein-induced lipid accumulation and inflammatory response via targeting toll-like receptor 4, 585 (6) (2011) 854–860.
[37] T.J. Schall, et al., A human T cell-specific molecule is a member of a new gene family, 141 (3) (1988) 1018–1025.
[38] G. McCormack, et al., Tissue cytokine and chemokine expression in inflammatory bowel disease, 50 (10) (2001) 491–495.
[39] A. Ben-Baruch, Inflammation-associated immune suppression in cancer: the roles played by cytokines, chemokines and additional mediators, Semin. Cancer Biol. 16 (1) (2006) 38–52.
[40] E.P. Caballero, et al., Endogenous osteopontin induces myocardial CCL5 and MMP-2 activation that contributes to inflammation and cardiac remodeling in a mouse model of chronic Chagas heart disease, 1864 (1) (2018) 11–23.
[41] J. Guo, et al., MicroRNA-1247 inhibits lipopolysaccharides-induced acute pneumonia in A549 cells via targeting CC chemokine ligand 16, 104 (2018) 60–68.