Long Non-Coding RNA (IncRNA) CRNDE Regulated Lipopolysaccharides (LPS)-Induced MRC-5 Inflammation Injury Through Targeting MiR-141

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Background: Pneumonia is a common disease with high morbidity and even death. In our country, pneumonia is the leading cause of child death. Therefore, research on the pathogenesis of pneumonia can help improve the treatment of pneumonia. Long non-coding RNA (lncRNA) is an important regulator of disease development, and its regulatory mechanism is closely related to cellular processes. However, the function and regulatory network of lncRNA is not fully elucidated in pneumonia.

Material/Methods: Quantitative real-time polymerase chain reaction (qRT-PCR) was applied to detect the expression of CRNDE and miR-141 in lipopolysaccharides (LPS)-induced MRC-5 cells and pneumonia tissues. MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2-tetrazolium) assay was used to assess cell proliferation. Flow cytometry assay was performed to detect cell apoptosis in LPS-induced MRC-5 cells. Enzyme-linked immunosorbent assay and western blot were used to measure the levels of interleukin (IL)-1β, IL-6, and tumor necrosis factor (TNF)-α, respectively. In addition, luciferase reporter assay and RNA immunoprecipitation (RIP) assay were applied to prove the relationship between CRNDE and miR-141.

Results: In this study, we found that CRNDE expression was induced in LPS-induced MRC-5 cells and pneumonia tissues. Moreover, miR-141 expression was low in LPS-induced MRC-5 cells and was verified as a target miRNA of CRNDE by using luciferase reporter assay and RIP assay. The downregulation of CRNDE and upregulation of miR-141 promoted cell viability, inhibited cell apoptosis, as well as decreased the levels of IL-1β, IL-6, and TNF-α. Moreover, we demonstrated that si-CRNDE transfection increased cell viability and suppressed cell apoptosis and the levels of IL-1β, IL-6, and TNF-α, which were alleviated by anti-miR-141 transfection in LPS-induced MRC-5 cells.

Conclusions: In this study, we found that downregulation of CRNDE and upregulation of miR-141 inhibited cell apoptosis and inflammation response and promoted cell viability in LPS-induced MRC-5 cells. Low CRNDE expression increased cell growth and suppressed inflammation response, which was impaired by inhibition of miR-141. These results suggested that a novel therapeutic target was found in pneumonia treatment.

MeSH Keywords: Inflammation Mediators • MicroRNAs • Pneumonia, Aspiration

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Background

Pneumonia in children is mainly caused by inflammation of the lungs caused by bacteria and viruses; it is one of the main causes of infant and child death [1–3]. Due to children with pneumonia have fewer symptoms and higher infection rates, it is urgent to find effective diagnostic and treatment strategies. Interleukin (IL)-1β, IL-6, and tumor necrosis factor (TNF)-α are very important pro-inflammatory factors that release apoptosis and necrosis, accumulate leukocytes, and inhibit microvascular function [2,4–6]. Regulatory factors related to the inflammatory response to pneumonia have important implications for the diagnosis and treatment of pneumonia.

Long non-coding RNA (lncRNA) is a type of non-coding RNA of more than 200 nucleotides in length [7]. As an important regulator, lncRNA is involved in the physiological and pathological processes of cells and tissues [8,9]. Differentially expressed lncRNA is closely related to cell proliferation, apoptosis, migration, invasion, drug resistance, and inflammatory and immune response in different diseases, such as cancer and pneumonia [10–14]. In pneumonia, the regulation mechanism of lncRNA plays an indispensable role; for example, lncRNA HAGLROS was shown to be involved in cell autophagy and apoptosis of pneumonia through regulating the miR-100/NF-κB axis [15]. CRNDE is also related to the production and development of various diseases and is highly expressed in renal cell carcinoma, cervical cancer, and bladder cancer [16–19]. Similarly, the expression of CRNDE in pneumonia increases significantly, indicating that CRNDE is also involved in some physiological and pathological processes of pneumonia, including inflammatory response and cell growth [20]. However, the function and regulatory network of CRNDE has not been fully elucidated in pneumonia.

In this study, we used lipopolysaccharides (LPS) to induce MCR-5 cell line to construct a pneumonia model, mainly to study the regulatory function and regulatory network of CRNDE in pneumonia, aiming to analyze the important role of CRNDE regulation mechanism in pneumonia cell progression and inflammatory response.

Material and Methods

Patients and sample collection

This experiment was approved by the Ethics Committee of The Fifth Affiliated Hospital of Sun Yat-sen University. A total of 35 children (17 males and 18 females; mean age±standard deviation, 10.6±2.54 years) with pneumonia were recruited to the present study. We exclude patients with other complications or patients who had received anti-inflammatory treatment. In addition, 15 gender-matched children (7 males and 8 females; mean age±standard deviation, 10.6±2.54 years) with fever were included as the control group. All children were from The Fifth Affiliated Hospital of Sun Yat-sen University. Their sera were collected, then centrifuged and stored in −80°C. Informed consent was provided by all patients and/or their guardians.

Cell culture and LPS treatment

Normal human embryo lung cell line MRC-5 was obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA) and incubated in Dulbecco’s modified Eagle’s medium (DMEM; Sigma-Aldrich, St Louis, MO, USA) containing 10% fetal bovine serum, 1% penicillin/streptomycin (Invitrogen), and then maintained in a humidified atmosphere with 5% CO₂ at 37°C. Different concentrations of LPS (5, 10, and 20 μg/mL, Sigma, purified by phenol extraction and gel filtration) was added into medium for 12 hours to construct pneumonia model.

Cell transfection

The LPS-induced MRC-5 cells were seeded into 6-well plates and incubated in DMEM overnight. Si-CRNDE, pc-CRNDE (CRNDE), miR-141, anti-miR-141 and their negative controls (si-NC, pcDNA, miR-NC, anti-miR-NC) were purchased from Ribobio (Guangzhou, China). All plasmids and oligos were transfected into LPS-induced MRC-5 cells using Lipofectamine 2000 Reagent (Life Technologies Corporation, Grand Island, NY, USA).

Quantitative real-time polymerase chain reaction (qRT-PCR)

Total RNA was isolated from serum and cells using TRIzol reagent (Invitrogen). cDNA of miR-141 was reverse transcribed with TaqMan miRNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA, USA). cDNA of CRNDE was reverse transcribed with Reverse Transcription Reagents (Applied Biosystems). U6 and glyceraldehyde phosphate dehydrogenase (GAPDH) were normalized against an internal control for miR-141 and CRNDE, respectively. Real-time polymerase chain reaction (RT-PCR) was performed using SYBR Green Real-time PCR analysis (Takara) according to the manufacturer’s protocol. The 2−ΔΔCt method was applied to calculate the relative expression of miR-141 and CRNDE.

The primer sequences:

- lncRNA CRNDE, forward, 5'-TGAAGAAAGGAAGGTGGCA-3' and reverse, 5'-TCAAGCCCATCTCACAAGA-3';
- GAPDH, forward, 5'-TGTCCGCTATGGTGGAAC-3' and reverse, 5'-ATTGGCATGCCTGTGCTCAT-3';
- miR-141, forward, 5'-AAGACGTACTCAGGCCATGTCC-3' and reverse, 5'-ATGGCATGGACTGTGGTCAT-3';
- CRNDE, forward, 5'-TGAAGGAAGGAAGTGTCAC-3' and reverse, 5'-AACGTTCTAGATGGTGTG-3'.
Western blot

Transfected cells were washed with phosphate-buffered saline (PBS) and extracted by re-suspending cell pellets in RNA immunoprecipitation assay (RIPA) buffer (Beyotime, Haimen, China). The proteins were separated using 10% sodium dodecyl sulfate (SDS)–polyacrylamide gel electrophoresis and transferred onto polyvinylidene difluoride (PVDF) membranes (Bio-Rad Laboratories, Inc.). The membranes were incubated with the primary antibodies against IL-1β, IL-6, TNF-α and GAPDH (1: 2000 dilution, Santa Cruz Biotechnology, Santa Cruz, CA, USA) at 4°C overnight. Subsequently, the membranes were incubated with horseradish peroxidase (HRP)-conjugated goat anti-rabbit secondary antibody (1: 2000 dilution, Santa Cruz Biotechnology) for 1 hour. The blot was detected by using an ECL Plus Western Blotting Substrate (Thermo Fisher Scientific, Inc., Waltham, MA, USA).

MTT (3-(4,5-dimethylthiazol-2-yl)-2 5-diphenyl-2-tetrazolium bromide) assay

Cell viability was measured using MTT assay. Briefly, 2×10^4 LPS-induced MRC-5 cells were seeded into a 96-well plate and incubated overnight. Then 10 μl MTT solution (Sigma-Aldrich, St. Louis, MO, USA) was added into plates and cultured for 4 hours at room temperature. We removed the supernatant after centrifugation and added 150 μl of dimethyl sulfoxide. The optical density (OD) was measured with a microplate reader (Bio-Rad, Hercules, CA, USA) at a wavelength of 450 nm.

Flow cytometry

Annexin V FITC/propidium iodide (PI) staining assay (Invitrogen, CA, USA) was applied to detect cell apoptosis according to the manufacturer’s instruction. Cells were harvested and washed in PBS. Next, each plate was stained with FITC-Annexin V and PI (BD Biosciences, San Jose, CA, USA) for 40 minutes. Flow cytometry (BD Biosciences) was used to detect cell apoptosis.

Luciferase reporter assay

The 3′-UTR of lncRNA CRNDE (containing miR-141 binding sites) or the mutation of CRNDE (containing mutation sites) was synthesized using PCR and inserted into pMIR-REPORT™ (ThermoFisher Scientific) to construct CRNDE wild-type reporter vector (CRNDE-WT) or CRNDE mutation reporter vector (CRNDE-MUT). CRNDE-WT or CRNDE-MUT and miR-NC or miR-141 were transfected into MRC-5 cells, respectively. After transfection for 48 hours, cells were harvested for measuring the luciferase activity using the Dual-Luciferase Reporter Assay System (Promega, Madison, WI, USA) according to the manufacturer’s instruction.

RNA immunoprecipitation assay and enzyme-linked immunosorbent assay (ELISA)

Magna RIP RNA-binding protein immunoprecipitation kit (Millipore, Billerica, MA, USA) was used to measure the enrichment of CRNDE in cells. Transfected cells were incubated with the Ago2 antibody (Abcam, Cambridge, MA, USA). Normal mouse IgG and miR-NC transfection act as negative and positive controls, respectively.

Cell collected and the inflammatory cytokines (IL-1β, IL-6, and TNF-α) were measured by using enzyme-linked immunosorbent assay (ELISA) kits (R&D Systems, Minneapolis, MN, USA) following the manufacturer’s instructions.

Statistical analysis

All data were presented as mean±standard deviation (SD) and all experiments were repeated at least 3 times. The data were analyzed and displayed using GraphPad Prism 7.0. Student’s t-test or one-way ANOVA were used to compare between groups. * P<0.05 was considered as statistically significant.

Results

LncRNA CRNDE was induced in pneumonia and LPS-induced MRC-5 cells

To further explore the expression of CRNDE in pneumonia, 15 healthy samples and 30 pneumonia samples were collected. qRT-PCR determined that the expression of CRNDE in pneumonia was higher than healthy controls (Figure 1A). Otherwise, LPS was used to construct the pediatric pneumonia model. Different concentrations of LPS (5, 10, and 20 μg/mL) were used to induced MRC-5 cells. The results showed that with the increase of LPS concentration, CRNDE expression gradually increased, especially in the 10 μg/mL and 20 μg/mL of LPS (P<0.01) (Figure 1B). We selected 10 μg/mL of LPS to use in further experiments. Thus, lncRNA CRNDE was induced in pneumonia and LPS-induced MRC-5 cells.

Si-CRNDE promoted cell growth and inhibited inflammation response in LPS-induced MRC-5 cells

To further explore the function of CRNDE in pneumonia, si-CRNDE was transfected into LPS-induced MRC-5 cells, which obviously inhibited CRNDE expression (Figure 2A). As shown in Figure 2B–2I, we found that LPS suppressed cell viability, induced cell apoptosis, and promoted the levels of IL-1β, IL-6, and TNF-α in MRC-5 cells. However, cell apoptosis in LPS+si-CRNDE group was higher than that in LPS+si-NC group. Moreover, si-CRNDE transfection inhibited cell apoptosis
and reduced the levels of IL-1β, IL-6, and TNF-α in LPS-induced MRC-5 cells. These results showed that si-CRNDE could alleviate the effects of LPS on MRC-5 cells. Thus, si-CRNDE promoted cell growth and inhibited inflammation response in LPS-induced MRC-5 cells, implying that downregulation of CRNDE could alleviate cell inflammation and injury in pneumonia.

**MiR-141 was a target of CRNDE**

To further investigate the regulatory network of CRNDE, miR-141 was predicted as a potential target of CRNDE using DIANA Tool (Figure 3A). To ensure the relationship between CRNDE and miR-141, CRNDE-WT and CRNDE-MUT were transfected into miR-141 or miR-NC transfection MRC-5 cell. The results of the luciferase reporter assay showed that luciferase activity was significantly reduced when miR-141 was bound to CRNDE-WT, but not CRNDE-MUT, indicating that miR-141 was a target of CRNDE (Figure 3B). RIP assay was used to further verify the direct binding between miR-141 and CRNDE. As shown in Figure 3C, compared with the miR-NC group, the expression of CRNDE was obviously enriched in the miR-141 groups. In addition, CRNDE transfection inhibited miR-141 expression while si-CRNDE transfection induced miR-141 expression in MRC-5 cells (Figure 3D). These results indicated that miR-141 was a target of CRNDE and was regulated by CRNDE.

**MiR-141 was decreased in pneumonia and LPS-induced MRC-5 cells**

To analyze the expression of miR-141 in pneumonia and LPS-induced MRC-5 cells, qRT-PCR was applied to detect its expression. As shown in Figure 4A, miR-141 expression was significantly inhibited in pneumonia. Additionally, with the increase of LPS, the expression of miR-141 was gradually decreased in MRC-5 cells, suggesting that LPS inhibited miR-141 expression in MRC-5 cells (Figure 4B).

**Overexpression of miR-141 contributed to cell growth and suppressed inflammation response in LPS-induced MRC-5 cells**

The function of miR-141 in pneumonia was investigated by transfecting miR-141 into the LPS-induced MRC-5 cells, which expressed high levels of miR-141 (Figure 5A). As shown in Figure 5B and 5C, miR-141 transfection promoted cell viability and inhibited cell apoptosis in LPS-induced MRC-5 cells. Otherwise, the levels of IL-1β, IL-6, and TNF-α were measured using ELISA and western blot. The results showed that overexpression of miR-141 decreased the levels of IL-1β, IL-6, and TNF-α (Figure 5D–5I). Briefly, overexpression of miR-141 contributed to cell growth and suppressed inflammation response in LPS-induced MRC-5 cells.

**Downregulation of CRNDE promoted cell growth and inhibited inflammation response in LPS-induced MRC-5 cells, which impaired by inhibition of miR-141**

Rescue experiments were applied to determine the regulatory network of CRNDE and miR-141 in LPS-induced MRC-5 cells. As shown in Figure 6, compared with the si-NC group, the expression of miR-141 and cell viability were remarkably induced, while cell apoptosis and the levels of IL-1β, IL-6, and TNF-α were significantly reduced in LPS-induced MRC-5 cells. However, the expression of miR-141 and cell viability were lower in the si-CRNDE+anti-miR-141 group than the si-CRNDE+anti-miR-NC group. Cell apoptosis and the levels of IL-1β, IL-6, and TNF-α were higher in the si-CRNDE+anti-miR-141 group than the si-CRNDE+anti-miR-NC group. These results determined...
that anti-miR-141 could alleviate the effect of si-CRNDE on LPS-induced MRC-5 cells and also indicated that CRNDE affected cell growth and inflammation response in LPS-induced MRC-5 cells by targeting miR-141.

Discussion

In this study, we found that the CRNDE/miR-141 axis has potential regulatory functions in pediatric pneumonia. To demonstrate the effects of CRNDE and miR-141 in pneumonia, we induced MRP-5 cells to construct a pneumonia model using LPS; we then studied their function in pneumonia by reducing the expression of CRNDE and increasing the expression of miR-141. We found that the effects of lowering CRNDE and increasing miR-141 expression in pneumonia were similar, both promoting cell growth and alleviating inflammatory responses. Moreover, both luciferase reporter assay and RIP assay demonstrated that miR-141 was a target miRNA of CRNDE. Furthermore, the rescue experiments also demonstrated that CRNDE affects cell growth and inflammation in pneumonia by regulating miR-141. Our results show that the CRNDE/miR-141 axis is involved in the development and inflammatory response of pneumonia.

Recently, many studies have shown that lncRNA plays an important role in disease production, and physiological and biochemical mechanisms. It has been reported that many differentially
Figure 3. MiR-141 was a target of CRNDE. (A) Putative miR-141 binding sequence and mutation sequence of CRNDE. (B) Luciferase reporter assay was used to verify that miR-141 was a target of CRNDE. (C) RIP assay was applied to ensure CRNDE could bind to miR-141. (D) The expression of miR-141 was detected in pcDNA, CRNDE, si-NC, and si-CRNDE groups by qRT-PCR. * \( P < 0.05 \), ** \( P < 0.01 \), and *** \( P < 0.001 \). RIP assay – RNA immunoprecipitation; qRT-PCR – quantitative real-time polymerase chain reaction.

Figure 4. MiR-141 was decreased in pneumonia and LPS-induced MRC-5 cells. (A) The expression of miR-141 was detected in pneumonia by qRT-PCR. (B) The expression of miR-141 was detected in different concentration LPS (5, 10, 20 μg/mL) by qRT-PCR. * \( P < 0.05 \), ** \( P < 0.01 \), and *** \( P < 0.001 \). LPS – lipopolysaccharide; qRT-PCR – quantitative real-time polymerase chain reaction.
expressed lncRNAs are involved in biological metabolism and tissue transfer of cells, especially in cancers [21–24]. For example, lncRNA MALAT1 is involved in the cellular processes of colon cancer, including apoptosis, migration, proliferation and invasion [25–28]. More than that, lncRNA could be used as a biomarker for the diagnosis and treatment of diseases [29,30]. Generally, lncRNA targets miRNA formation regulatory networks were involved in various physiological and biochemical processes of cells in diseases [35–38]. Therefore, miR-141 was verified to be a target miRNA for CRNDE in this experiment. In addition, miR-141 is also involved in cellular processes in many diseases, including cancers and pneumonia [39–42]. The study of Wang et al. have shown that miR-141, as a tumor suppressor, participated in glioma growth through targetting ATF5 [43]. In this experiment, miR-141 expression was low in pneumonia, and the effects of high miR-141 expression on cells were consistent with the effect of reducing CRNDE expression on cells, indicating that CRNDE negatively regulates miR-141 in pneumonia.

When pneumonia is produced, inflammatory factors are released, which could promote apoptosis and inhibit cell

Figure 5. Overexpression of miR-141 contributed to cell growth and suppressed inflammation response in LPS-induced MRC-5 cells. (A) The expression of miR-141 was measured in control, LPS, LPS+miR-NC, and LPS+miR-141 groups by qRT-PCR. (B) Cell viability was detected in control, LPS, LPS+miR-NC, and LPS+miR-141 groups by MTT assay. (C) Cell apoptosis was detected in control, LPS, LPS+miR-NC, and LPS+miR-141 groups by flow cytometry. (D-F) The levels of IL-1β (D), IL-6 (E), and TNF-α (F) were measured in control, LPS, LPS+miR-NC, and LPS+miR-141 groups by ELISA assay. (G-I) The levels of IL-1β (G), IL-6 (H), and TNF-α (I) were detected in control, LPS, LPS+miR-NC, and LPS+miR-141 groups with western blot assay. * P<0.05. ** P<0.01, and *** P<0.001. LPS – lipopolysaccharide; qRT-PCR – quantitative real-time polymerase chain reaction; MTT – 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2-tetrazolium; IL – interleukin; TNF – tumor necrosis factor; NC – negative control; ELISA – enzyme-linked immunosorbent assay.
proliferation [44,45]. Relief of the inflammatory response contributes to pneumonia treatment. In this study we found that CRNDE-reduced expression and miR-141-enhanced expression could suppress inflammatory factors, suggesting CRNDE and miR-141 also play an important role in the treatment of pneumonia.

Notably, in this study we demonstrated for the first time that miR-141 was a target miRNA of CRNDE, which could further improve the regulatory network of CRNDE in pneumonia, providing a new target site for the treatment of pneumonia, and consolidating the important role of CRNDE in the diagnosis of pneumonia. In addition, accumulating evidence indicates that miR-141 can regulate the physiological mechanisms by which

Figure 6. Downregulation of CRNDE promoted cell growth and inhibited inflammation response in LPS-induced MRC-5 cells, which were impaired by inhibition of miR-141. (A) The expression of miR-141 was measured in control, LPS, LPS+si-NC, LPS+si-CRNDE, LPS+si-CRNDE+anti-miR-NC, and LPS+si-CRNDE+anti-miR-141 groups by qRT-PCR. (B) Cell viability was detected in control, LPS, LPS+si-NC, LPS+si-CRNDE, LPS+si-CRNDE+anti-miR-NC, and LPS+si-CRNDE+anti-miR-141 groups by MTT assay. (C) Cell apoptosis was detected in control, LPS, LPS+si-NC, LPS+si-CRNDE, LPS+si-CRNDE+anti-miR-NC, and LPS+si-CRNDE+anti-miR-141 groups by flow cytometry. (D-F) The levels of IL-1β (D), IL-6 (E), and TNF-α (F) were measured in control, LPS, LPS+si-NC, LPS+si-CRNDE, LPS+si-CRNDE+anti-miR-NC, and LPS+si-CRNDE+anti-miR-141 groups by ELISA assay. (G-I) The levels of IL-1β (G), IL-6 (H), and TNF-α (I) were detected in control, LPS, LPS+si-NC, LPS+si-CRNDE, LPS+si-CRNDE+anti-miR-NC, and LPS+si-CRNDE+anti-miR-141 groups with western blot assay. * P<0.05, ** P<0.01, and *** P<0.001. LPS – lipopolysaccharide; qRT-PCR – quantitative real-time polymerase chain reaction; MTT – 3-(4,5-dimethylthiazol-2-yi)-2,5-diphenyl-2-tetrazolium; IL – interleukin; TNF – tumor necrosis factor; ELISA – enzyme-linked immunosorbent assay.
downstream target genes affect cell [46–48]. For example, in hepatocellular carcinoma cells and ovarian cancer cells, the miR-141/KEAP1 axis was shown to be involved in the mechanism of cell growth and chemoresistance [46,48]. Therefore, we speculate that CRNDE affected miR-141 expression and further influenced downstream target genes of miR-141 to regulate the biochemical mechanisms of cells, which will be the focus of our next research.

Conclusions

In this study, we verified the function of the regulatory network in LPS-induced MRC-5 cell, and we improved our understanding of the regulatory network of CRNDE in pneumonia, however, these results require validation in in vivo experiments. In future research we plan to verify the function of this regulatory network in mice and further explore downstream regulatory mechanisms of CRNDE, hoping to further elucidate the regulatory mechanisms and regulatory networks of CRNDE in pneumonia.

Conflicts of interest

None.

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