Long non-coding RNA NKILA weakens TNF-α-induced inflammation of MRC-5 cells by miR-21 up-regulation

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Abstract

Background: Infantile pneumonia (IP) seriously affects the health of children. This article mainly discussed the protective effect of long non-coding RNA NKILA (lnc NKILA) on IP by detecting cell viability, apoptosis and inflammatory response of MRC5 cells.

Methods: Cell counting kit-8 (CCK-8) was used to detect cell viability, while flow cytometry was used to detect cell apoptosis. The expression of apoptosis-associated factors (Bcl-2, Bax, PARP and Cleaved-PARP) and NF-κB and JNK pathway-related factors (t-IκBα, p-IκBα, t-p65, p-p65, β-actin, t-JNK and p-JNK) were tested by western blot. Otherwise, productions of inflammatory factors interleukin (IL)-1β and IL-6 were tested by enzyme-linked immunosorbent assay (ELISA) and western blot. Furthermore, RNA levels were respectively tested and changed by RT-qPCR and cell transfection.

Results: Tumour necrosis factor-α (TNF-α) treatment reduced cell viability, induced cell apoptosis and promoted inflammatory factors expression. NKILA overexpression remitted TNF-α-induced injury. Moreover, NKILA positively regulated miR-21. miR-21 inhibition could weaken the functions of NKILA overexpression on TNF-α-induced injury. At last, NKILA and miR-21 were involved in the regulation of JNK and NF-κB pathways.

Conclusions: NKILA overexpression remitted TNF-α-induced MRC5 cell injury by up-regulation of miR-21 and via inactivation of JNK and NF-κB signaling pathways.

Introduction

Pneumonia is a common respiratory disease in paediatrics [1]. Bacteria, viruses and mycoplasma are common pathogens that cause infantile pneumonia (IP) [2]. In recent years, with the changes in the environment and the abuse of antibiotics, the incidence of IP has continued to increase and the clinical symptoms are diverse [3,4]. If not treated in time during acute attack, it can cause serious damage to the respiratory system and other systems, even organ dysfunction [5]. At present, the pathogenesis of IP was not fully understood. Some studies have pointed out that excessive immune and inflammatory reactions may be important causes of IP [6,7]. Therefore, studying the mechanism of inflammatory response was of great significance for the treatment of IP. On the other hand, researchers found that non-coding RNA played an important role in pneumonia [8,9].

Long non-coding RNA (LncRNA) is a type of functional RNA molecule that does not have the ability to encode proteins between 200 and 100,000 nt. It plays an important role in gene expression, RNA splicing and translation, and so on [10]. There are various examples of IncRNAs that were aberrant expressed in IP, like Inc GAS5 [11] and Inc CRNDE [12]. LncRNA NKILA (NKILA) was an IncRNA gene that interacted with NF-κB and located at 20q13.31 [13]. Moreover, a previous study proposed that NKILA was highly expressed in ankylosing spondylitis and played an inhibiting role in inflammation [14]. Although many studies found that NKILA inhibited inflammation, its specific mechanism of action was still not fully understood.

MicroRNAs (miRNAs) are another type of endogenous non-coding RNAs, approximately 20 nt in length [15]. It can accumulate in cells and specifically regulate gene expression by binding to the 3′ untranslated region (3′UTR) of its target gene, and thereby regulating gene transcription [9]. It is very important for maintaining the biological activity of cells. Similarly, there are a large number of abnormally expressed miRNAs in IP, such as miR-21, miR-26a [16]. miR-21 was located at chromosome 17q23.1 and was widely distributed in human cells and tissues to regulate the expression of various proteins [17]. Many studies found that miR-21 was highly expressed in IP [16] and played inhibitory roles in inflammation [18], so we speculate that it may be a potential therapeutic target of IP.

In this study, human lung fibroblast MRC-5 cells were selected as the study object. Tumour necrosis factor-α (TNF-α) treatment was conducted to establish the inflammatory model of IP. The roles and possible molecular mechanisms of LncRNA and miRNA which may be related to pneumonia were screened by software and were explored in this study. There is hope to provide a theoretical basis for future treatment of IP.
Materials and methods

Cell culture and treatment

MRC5 cells (laboratory storage) were reconstituted in Dulbecco’s modified Eagle medium (DMEM, Beyotime, Shanghai, China). Besides, the DMEM was supplemented with 10% foetal bovine serum (FBS; Hyclone, Logan, UT, USA) and cells were cultured under 37 °C, 5% CO₂ and sterile conditions. Moreover, an inflammatory injury model of MRC5 cells were established by conducting TNF-α (20 ng/mL, Sigma, St. Louis, MO) treatment for 24 h [19].

Cell counting kit-8 (CCK-8) assay

MRC5 cells (1 × 10⁶ cells/well) were seeded in 96-well plates (Corning, Beijing, China). When cell coverage was reached about 80%, it was processed according to the experimental needs. Each group had six duplicate wells. After adding 10 µL of CCK-8 (Beyotime, Haimen, China) to each well, plates were incubated at 37 °C for 1 h. The absorbance of each well was recorded by using a microplate reader (λ = 450 nm, Bio-Rad, Hercules, CA, USA). The average of the absorbance values of the six wells was taken, and calculation of the cell viability was performed according to the following formula:

\[
\text{Cell viability} = \frac{OD_{\text{treatment group}} - OD_{\text{control}}}{OD_{\text{no treatment control group}} - OD_{\text{control}}} \times 100\%
\]

Flow cytometry

The cell apoptosis (1 × 10⁵ cells/well) was tested with the help of Annexin V-FITC/PI kit (Beyotime, Shanghai, China). Cells were washed for three times with PBS. Trypsin (Beyotime, Shanghia, China) was used for cell collection. 500 µL of the Binding Buffer was mixed with the cell suspension. Then, 5 µL of Annexin V-FITC was added and maintained for 5 min, subsequently 5 µL of PI was added and reacted for 10 min at room temperature in the dark. The apoptosis level was measured by using flow cytometry (Beckman Coulter, Miami, FL, USA).

Enzyme-linked immunosorbent assay (ELISA)

The ELISA kit was purchased from Abcam (Cambridge, MA) and the experiment was carried out in strict accordance with the instructions. 100 µL of cell supernatant was used to detect the levels of interleukin (IL)-1β (ab100562) and IL-6 (ab178013) by double antibody sandwich method. After coating, the simples, enzyme-labelled antibody and substrate liquid were respectively added, the OD values were detected on the microplate reader (Molecular Devices Corp., Sunnyvale, CA, USA) at 450 nm.

Transfection

MiR-21 inhibitor and the negative control (NC) were synthesized by GenePharma Co. (Shanghai, China) and were transfected into cells by using lipofectamine 3000 (Life Technologies Corporation, Camarillo, CA, USA). The full-length of NKILA sequences was constructed into pcDNA3.1 vector and the recombined vector was renamed as pc-NKILA. Lipofectamine 3000 was also used in this experiment. The medium (0.5 mg/mL G418, Sigma, St. Louis, MO, USA) was used to select stably transfected cells.

Quantitative reverse transcription polymerase chain reaction (qRT-PCR)

The experiment was performed following qRT-PCR kit instructions. Trizol (Solarbio, Shanghai, China) was used to extract total RNA. The cDNA was reverse transcribed and qRT-PCR was performed by using EmeraldAmp™ PCR Master Mix Kit (TaKaRa, Kusatsu, Japan) and random hexamers or oligo (dT). The total PCR reaction system was 10 µL and reverse transcription conditions were 25 °C (10 min), 48 °C (30 min) and 95 °C (5 min). GAPDH was an internal reference gene of IncRNA while U6 was an internal reference gene of miRNA.

Western blot

The total protein was extracted by using the total extraction sample kit (Solarbio, Shanghai, China) and the protein was quantified by using BCA method (Pierce, WI, USA). Separation gel (10%) and concentrated gel (5%) were prepared. Each well was added with 20 µL of protein sample. The electrophoresis was 80 V until bromophenol blue reached the edge of the concentrated gel. Then, we adjusted the voltage to 120 V. Electrophoresis was finished when bromophenol blue reached the bottom of the gel. The nitrocellulose membrane (Millipore, Billerica, USA) transferring was conducted at 3 mA for 2 h. The membrane was blocked by 5% bovine serum albumin (BSA; Solarbio, Shanghai, China) at room temperature for 2 h. The primary antibody was diluted (1:1000) and incubated at 4 °C overnight. Then, it was combined with secondary antibody (1:5000) for 2 h at room temperature. After ECL chemiluminescence for 30 min, Image-Pro Plus (Bio-Rad, Shanghai, China) analyzed protein content. β-Actin was an internal reference. The primary antibodies included Bcl-2 (ab182858), Bax (ab182733), PARP (ab74290), Cleaved-PARP (ab32561), IL-1β (ab9722), IL-6 (ab233551), t-IκBα (ab7217), p-IκBα (ab133462), t-p65 (ab32536), p-p65 (ab86299), β-actin (ab115777), t-JNK (ab247935) and p-JNK (ab47337). The secondary antibody was HRP-tagged donkey anti-rabbit antibody (ab6802). All antibodies were from Abcam (Cambridge, MA, USA).

Statistical analysis

SPSS 16.0 statistical software was used to analyze the data (SPSS, Chicago, IL, USA). The data were expressed as mean ± standard deviation (SD). All trials possess at least three experiments. One-way analysis of variance (ANOVA) was used for multiple comparisons, while group comparison used Student’s t-test. \( p < .05 \) was considered as statistically significant.

Results

The TNF-α inflammatory injury model was established

Cell viability, cell apoptosis, apoptosis-related proteins and inflammatory cytokines were respectively detected to determine whether the TNF-α inflammatory injury model was successfully established. It was shown that TNF-α remarkably
reduced cell viability \((p < .01, \text{Figure 1(A)})\) but improved cell apoptosis \((p < .001, \text{Figure 1(B)})\). Meanwhile, apoptosis-related Bcl-2 expression was decreased, while Bax and Cleaved-PARP expression was increased in TNF-\(\alpha\)-induced cells \((\text{all } p < .001, \text{Figure 1(C,D)})\). Further, ELISA and western blot results discovered that inflammatory cytokines IL-1\(\beta\) and IL-6 expression was remarkably up-regulated \((\text{both } p < .01, \text{Figure 1(E,F)})\). In conclusion, the TNF-\(\alpha\) inflammatory model was successfully established.

**TNF-\(\alpha\)-induced injury was remitted by NKILA overexpression**

To determine the effect of NKILA on IP, we overexpressed NKILA in MRC5 cells. It turned out to be that when pc-NKILA was transfected into MRC5 cells, NKILA expression was notably up-regulated \((p < .001, \text{Figure 2(A)})\). Besides, cell viability was remarkably increased \((p < .05, \text{Figure 2(B)})\) and apoptosis was reduced \((p < .01, \text{Figure 2(C)})\) after NKILA overexpression. Meanwhile, apoptosis-related Bcl-2 expression was increased, while Bax and Cleaved-PARP expression was reduced \((\text{all } p < .01, \text{Figure 1(D,E)})\). Further ELISA and western blot results discovered that inflammatory cytokines IL-1\(\beta\) and IL-6 expression was remarkably down-regulated \((\text{both } p < .01, \text{Figure 1(F,G)})\). In conclusion, TNF-\(\alpha\)-induced injury was remitted by NKILA overexpression.

**NKILA overexpression up-regulated miR-21 expression**

In order to further understand the regulating mechanism of NKILA in IP, we further explored the expression of miR-21 in NKILA overexpressed MRC5 cells. Fortunately, we found that compared to pcDNA3.1 transfected group, miR-21 level was remarkably enhanced in pc-NKILA transfected group \((p < .01, \text{Figure 3})\), indicating that miR-21 expression was positively correlated with NKILA expression.
NKILA overexpression-induced protective effects were weakened by miR-21 inhibition

Undertaking Figure 3, we transfected miR-21 inhibitor into MRC5 cells and results showed that miR-21 expression was remarkably decreased \( (p < .001, \text{Figure 4(A)}) \). Based on this, miR-21 inhibitor was transfected into NKILA overexpressed cells, and following results showed that NKILA overexpression-triggered effects on cell viability and cell apoptosis were both notably remitted by miR-21 inhibition (both \( p < .05 \), Figure 4(B,C)). Meanwhile, apoptosis-related Bcl-2 expression was increased, while Bax and Cleaved-PARP expression was reduced. (F, G) Inflammatory cytokines interleukin (IL)-1\(\beta\) and IL-6 expression were remarkably up-regulated. \( *p < .05; **p < .01; ***p < .001.\)

**Figure 2.** Overexpression of NKILA remitted tumour necrosis factor-\(\alpha\) (TNF-\(\alpha\))-induced injury of MRC5 cells. (A) pc-NKILA transfection meaningfully increased NKILA expression. On this basis, when pc-NKILA was transfected into TNF-\(\alpha\)-injured cells, (B) cell viability was remarkably increased and (C) apoptosis was reduced. Meanwhile, (D, E) apoptosis-related Bcl-2 expression was increased, while Bax and Cleaved-PARP expression was reduced. (F, G) Inflammatory cytokines interleukin (IL)-1\(\beta\) and IL-6 expression were remarkably down-regulated. \( *p < .05; **p < .01; ***p < .001.\)

**Figure 3.** NKILA positively regulated miR-21 expression. When pc-NKILA was transfected into MRC5 cells, miR-21 level was remarkably enhanced. \( **p < .01.\)

**NKILA overexpression-induced protective effects were weakened by miR-21 inhibition**

Undertaking Figure 3, we transfected miR-21 inhibitor into MRC5 cells and results showed that miR-21 expression was remarkably decreased \( (p < .001, \text{Figure 4(A)}) \). Based on this, miR-21 inhibitor was transfected into NKILA overexpressed cells, and following results showed that NKILA overexpression-triggered effects on cell viability and cell apoptosis were both notably remitted by miR-21 inhibition (both \( p < .05 \), Figure 4(B,C)). Meanwhile, apoptosis-related Bcl-2 expression was increased, while Bax and Cleaved-PARP expression was reduced after miR-21 inhibition \( (p < .05 \text{ or } p < .01, \text{Figure 4(D,E)}) \). Further ELISA and western blot results discovered that inflammatory cytokines IL-1\(\beta\) and IL-6 expression was remarkably up-regulated \( (p < .01, \text{Figure 4(F,G)}) \). In conclusion, NKILA overexpression-induced protective effects were weakened by miR-21 inhibition.
NKILA overexpression inhibited NF-κB and JNK pathways by modulating miR-21 expression

At first, TNF-α treatment remarkably increased the ratios of p/IκBα, p/p65 (both p < .001, Figure 5(A,B)) and p/t-JNK (p < .001, Figure 5(C,D)). After pc-NKILA was transfected into TNF-α-injured cells, these ratios were reduced (all p < .01). Further, miR-21 inhibition remarkably recovered NKILA overexpression-triggered impacts (all p < .05). In conclusion, NKILA overexpression inhibited NF-κB and JNK pathways by modulating miR-21 expression.

Discussion

IP is a common respiratory infection in infants and young children. It is an important feature accompanied by the
occurrence of inflammation [20]. TNF-α is one of the most important pro-inflammatory cytokines in the body. Endogenous TNF-α regulates immune cell function, mediates local and systemic inflammatory responses [21]. In this paper, we successfully constructed an inflammatory injury model using TNF-α treatment. On this basis, we found that NKILA and miR-21 play important roles in viability, apoptosis and the expression of inflammatory factors. Finally, we found that NKILA and miR-21 played a role through NF-κB and JNK pathways.

NKILA is an lncRNA that regulates the IκB/NF-κB pathway [22]. In recent years, the research explored the role of NKILA in cancer. Studies presented NKILA restrained nasopharyngeal carcinoma metastatic potential [23] and could be a diagnosis and prognosis marker of colorectal cancer [24]. Although the specific intrinsic mechanism had not been fully elucidated, studies have confirmed that NKILA played an important role in inflammation. For instance, Li et al. presented NKILA was up-regulated in diabetic cardiomyopathy and could restrain cell apoptosis [25]. Zhu et al. presented NKILA played a pro-inflammatory response role in endothelium inflammation [26]. In this IP model, we proved pc-NKILA remarkably increased TNF-α-induced cell viability and reduced apoptosis. Meanwhile, apoptosis cytokines Bcl-2 expression was increased while Bax and Cleaved-PARP expression were reduced. Inflammatory cytokines IL-1β and IL-6 expression were remarkably down-regulated (p < .01, Figure 1(F,G)). All these indicated NKILA exerted an anti-inflammatory effect in IP.

LncRNAs usually played a role by regulating miRNAs. Lnc NEAT1 and miR-124 were involved in sepsis [27]. Lnc THRIL enhanced LPS-injury by modulating miR-125b [28]. Moreover, Lnc SNHG16 targets worked in acute pneumonia by miR-146a-5p [29]. To further explore the intrinsic mechanisms which NKILA inhibited inflammation, we predicted miR-21 through bioinic software.

The miR-21 sequence is UGUCGGGUAGCUUAUCAGACUGAUGUUGACUGUUGAAUCUCAUGGCAACACCAGUGUGACUGACA [30]. It is widely distributed in human cells and tissues. Besides, it can regulate the expression of various proteins and has powerful biological functions [31]. Some scholars have pointed out that miR-21 could target PDCD4 (a pro-inflammatory protein) and inhibited the expression of PDCD4 protein [32]. miR-21 can also directly bind to the Toll-like receptor (TLR) family, inhibiting the pro-inflammatory effects of TLR8 [33]. Further studies found that miR-21 was involved in bleomycin-induced pulmonary fibrosis in alveolar macrophages [34]. Moreover, researchers displayed miR-21 increased the phosphorylation of IκBα, p65 and JNK in MRC5 cells. Tumour necrosis factor-α (TNF-α) remarkably increased phosphorylation levels of (A, B) IκBα, p65 and (C, D) JNK. NKILA overexpression reduced the phosphorylation. Further, miR-21 inhibition remarkably recovered NKILA overexpression-triggered effects. *p < .05; **p < .01; ***p < .001.
was up-regulated in pneumonia [35] and miR-21 could inhibit pneumonia [36]. In this paper, miR-21 also played an inhibiting role in inflammation. Besides, we discovered NKILA positively regulated miR-21. miR-21 inhibitor weakened NKILA-induced protection.

NF-κB acts as an important inflammatory pathway to regulate inflammation [37]. NKILA expression is activated by NF-κB pathway and directly occluded by interacting with the NF-κB/NF-κB complex. NF-κB phosphorylates the site, thereby inhibiting IκB phosphorylation and activation of NF-κB by IKK [22]. Furthermore, the study discovered miR-21 regulated the inflammatory response caused by bacterial infection by targeting JNK [38]. Moreover, it was found that the NF-κB and JNK pathways exerted an important part on IP [39].

Surprisingly, the same phenomenon was also displayed in this article. pc-NKILA reduced the TNF-α-induced phosphorylation levels of IκBα, p65 and JNK and miR-21 inhibitor remarkably recovered pc-NKILA-inducible phosphorylation.

Conclusions

This study focused on the protective effect of NKILA on IP, which may be due to inhibition of apoptosis and inflammatory response of MRC-5 cells via miR-21 and NF-κB/JNK pathways. Our experiments only did some basic research on NKILA, and further research is still in progress. This article further expanded the functions of NKILA and provided a new perspective for the treatment of IP.

Disclosure statement

No potential conflict of interest was reported by the authors.

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Data availability

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

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